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(54) Title: ANTI-CANCER DNA VACCINE EMPLOYING PLASMIDS ENCODING MUTANT ONCOPROTEIN ANTIGEN AND CALRETICULIN

(57) Abstract: Novel nucleic acid vectors comprising sequences encoding (a) calreticulin or a domain thereof, and (b) an antigen, such as human papillomavirus oncoproteins E7 or E6 in detoxified form, are disclosed, as are methods for using such vectors to induce antigen-specific immune responses and to treat or prevent development of tumors.

Anti-Cancer DNA Vaccine Employing Plasmids Encoding Mutant Oncoprotein Antigen and Calreticulin

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention in the fields of molecular biology, immunology and medicine relates to chimeric nucleic acid molecules that encode an antigen, optionally a signal peptide, and an immunogenicity-gptentiating polypeptide ("IPP") such as calreticulin (CRT), and their uses a immunogenic compositions to induce and enhance immune responses, primarily cytotoxic T lymphocyte responses to specific antigens such as tumor or viral antigens.

Description of the Background Art

Cytotoxic T lymphocytes (CTL) are critical effectors of anti-viral and antitumor responses (reviewed in Chen, CH *etal, J BiomedSci.* 5: 231-52, 1998; Pardoll, DM. *Nature Med 4:525-31,* 1998; Wang, RF *et al, Immunol Rev. 1*70: 85-100, 1999). Activated CTL are effector cells that mediate antitumor immunity by direct lysis of their target tumor cells or virus-infected cells and by releasing of cytokines that orchestrate immune and inflammatory responses that interfere with tumor growth or metastasis, or viral spread. Depletion of $CD8^+$ CTL leads to the loss of antitumor effects of several cancer vaccines (Lin, K-Y *et al..,* Cane Res. *56:21-6,* 1996; Chen, C-H *et al.,* Cane Res. *60:* 035-42, 2000). Therefore, the enhancement of antigen presentation through the MHC class I pathway to $CD8^+$ T cells has been a primary focus of cancer immunotherapy.

DNA vaccines have emerged as an attractive approach for antigen-specific cancer immunotherapy. DNA vaccines offer many advantages over more conventional vaccines, such as peptide or attenuated live pathogens. One advantage is that DNA vaccines are reasonably stable and can be easily prepared and harvested in large quantities. Additionally, naked plasmid DNA is relatively safe and can be repeatedly administered without adverse effects. Furthermore, because DNA is able to be maintained in cells for long-term expression of the encoded antigen, maintenance of immunologic memory is possible (see, Donnelly, JJ *et al, Annu Rev Immunol* 1997, 15:617-648; Pardoll, *supra;* Robinson, HL, *Vaccine* 75:85-787, 1997; Gurunathan, S *et al, Annu Rev Immunol* 18:927-74, 2000).

However, one limitation of these vaccines is their lack of potency, since the DNA vaccine vectors generally do not have the intrinsic ability to be amplified and to spread *in vivo* as do some replicating viral vaccine vectors. Furthermore, some tumor antigens such as the E7 protein of human papillomavirus- 16 ("HPV-1 6") are weak immunogens (Chen *et al.,* 2000, *supra).* Therefore, there is a need in the art for strategies to enhance DNA vaccine potency, particularly for more effective cancer and viral immunotherapy.

One strategy taken by the present inventors in the present invention to enhance the presentation of antigen through the MHC class I pathway to $CD8^+$ T cells is the exploitation of the features of certain polypeptides to target antigenic polypeptide to which they are fused. Such polypeptide are referred to collectively herein as "zmmunogenicity-gptentiating (or -promoting) polypeptide" or "IPP" to reflect this general property, even though these IPP's may act by any of a number of cellular and molecular mechanisms that may or may not share common steps. This designation is intended to be interchangeable with the term "targeting polypeptide." Inclusion of nucleic acid sequences that encode polypeptides that modify the way the antigen encoded by molecular vaccine is "received" or "handled" by the immune system serve as a basis for enhancing vaccine potency.

Calreticulin

Calreticulin (CRT), an abundant 46 kilodalton (kDa) protein located in the lumen of the cell's endoplasmic reticulum (ER), displays lectin activity and participates in the folding and assembly of nascent glycoproteins. See, *e.g.,* Nash., *MoI Cell Biochem* 735:71-8, 1994; Hebert, *J Cell Biol 139:613-*23, 1997; Vassilakos, A, *Biochemistry* 37:3480-90, 1998; Spiro, *J Biol Chem 271:1* 1588-94, 1996). Rabbit, human, mouse, and rat CRT's show >90% sequence identity (Michalak, M *et al, Biochem J, 344 Pt* 2:281-92, 1999).

CRT associates with peptides transported into the ER by transporters that are associated with antigen processing, such as TAP-I and TAP-2 (Spee , *Eur J Immunol* 27:2441-9, 1997). CRT also forms complexes with peptides *in vitro.* Upon administration to mice, these complexes, elicited peptide-specific CD8+ T cell responses (Basu, *J Exp Med* 759:797-802, 1999; Nair, *J Immunol 162:6426-32,* 1999). CRT purified from murine tumors elicited immunity specific for the tumor from which the CRT was taken, but not for an antigenically distinct tumor (Basu, *supra).* By pulsing mouse dendritic cells (DCs) *in vitro* with a CRT-peptide complex, the peptide was re-presented by MHC class I molecules on the DCs to stimulate a peptide-specific CTL response(Nair, *supra).*

The CRT protein is composed of three domains, the N-domain, P-domain and C-domain. The N-domain (residues 1-1 80), also known as vasostatin, is highly conserved among CRTs from different species (Krause, KH *et al, Cell* δδ;439-43, 1997). The N-domain interacts with the DNA-binding domain of the glucocorticoid receptor *in vitro,* with rubella virus RNA (Singh, NK *et al, Proc Natl Acad Sci USA* 97:12770-74, 1994) , with α-integrin (Rojiani, MV *et al., Biochemistry* 30:9859-66, 1991), and with protein disulphide-isomerase (PDI) and ER protein 57 (ERp57) (Corbett, EF *et al., J Biol Chem* 1999, *274:6203-11,* 1999). The N-domain of calreticulin also inhibits proliferation of endothelial cells and suppresses angiogenesis (Pike, SE *et al. J Exp Med* 7<5<°:2349-56, 1998). The P-domain (residues 181-280) is rich in Pro and contains two sets of three sequence repeats. This domain binds Ca^{++} with high affinity (Baksh, S *et al., J Biol Chem 266:21458-65,* 1991). The P-domain is thought to be critical

for the lectin-like chaperone activity of CRT (Vassilakos *et al, supra)* and also interacts with PDI and perforin (Andrin, C *et al, Biochemistry* 37:10386-94, 1998; Fraser, SA *et al, Biochem Cell Biol* 76:881-7, 1998). The C-terminal region of CRT is highly acidic and terminates with the KDEL ER retrieval sequence Michalak, M *et al, J Biol Chem* 277:29436-45, 1996). The C domain of CRT binds to Ca (Baksh *et al, supra)* and to blood-clotting factors (Kuwabara, K *et al, J Biol Chem* 270:8179-87, 1995) and inhibits injury-induced restenosis (Dai, E *et al, Arterioscler Thromb Vase Biol* 77:2359-68, 1997).

CRT also has anti-angiogenic effects. CRT and its N domain are endothelial cell inhibitors that can suppress tumor growth (Pike, SE, *Blood* 94:2461-8, 1999). Tumor growth and metastasis depend on the existence of an adequate blood supply. As tumors grow larger, adequate blood supply is often ensured by new vessel formation, a process termed angiogenesis. Therapeutic agents that target and damage tumor vasculature can prevent or delay tumor growth and even promote regression or dormancy.

Immunogenic Constructs with HPV E7 as a Model Antigen

The present inventors and their colleagues previously developed several intracellular targeting and intercellular spreading strategies to enhance DNA vaccine potency using various immunogenicity-potentiating polypeptide ("IPP"). See for example, publications of the present inventors and their colleagues: Hung, CF *et al, J Virol* 76:2676-82, 2002; Cheng, WF *et al., JClin Invest 108:669-78,* 2001; Hung, CF *et al, J Immunol 166:5733-40,* 2001; Chen, CH *et al, Gene Ther* (5:1972-81, 1999; Ji, H *et al, Hum Gene Ther* 70:2727-40, 1999; Chen, CH *et al, Cancer Res* 60:1035-42, 2000; U.S. Pat 6,734,173, WO 01/29233; WO03/085085; WO 02/012281; WO 02/061113).

Among these strategies was the linkage of linking antigen to the intracellular targeting moiety CRT. The present inventors and their colleagues were the first to provide naked DNA and self-replicating RNA vaccines that incorporated CRT (or other IPPs). The present inventors and their colleagues also demonstrated that linking antigen to *Mycobacterium tuberculosis* heat shock protein 70 (HSP70) or its C-terminal domain, domain II of *Pseudomonas aeruginosa* exotoxin A (ETA(dII)), or the sorting signal of the lysosome-associated membrane protein type 1 (Sig/LAMP-1) enhanced DNA vaccine potency compared to compositions comprising only DNA encoding the antigen of interest. To enhance MHC class II antigen processing, the present inventors' colleagues (Lin, KY *et al , Cancer Res 56:* 21-6, 1996) linked the sorting signals of the lysosome-associated membrane protein (LAMP-I) to the cytoplasmic/nuclear human papilloma virus (HPV-16) E7 antigen, creating achimera (Sig/E7/LAMP-1). These findings point to the importance of adding an "element" to an antigenic composition at the DNA level to enhance *in vivo* potency of a recombinant DNA vaccine.

Intradermal administration of DNA vaccines via gene gun *in vivo* have proven to be an effective means to deliver such vaccines into professional antigen-presenting cells (APCs), primarily dendritic

cells (DCs), which function in the uptake, processing, and presentation of antigen to T cells. The interaction between APCs and T cells is crucial for developing a potent specific immune response.

Various DNA constructs described by the present inventors or others in the prior art, have resulted in certain combinations that induced a heightened immune response in experimental animals. However, none of these vaccines have been ideally designed for use in humans where administration may be limited for practical or other reasons to intramuscular (i.m.) injection. Because direct transduction of professional APCs in muscle tissue is not likely to occur due to paucity of such cells in muscle. That leaves cross-priming as the most likely mechanism for the induction of heightened immunity in humans. Optimizing vaccine constructs for cross priming requires that an element be added that promotes the secretion of the expressed polypeptide antigenic moiety, preferably as a fusion polypeptide with a molecule that promotes antigen processing via the MHC class I pathway. Moreover, it best to used plasmid constructs that are know to be safe and effective in humans. Finally, in the case of HPV oncoprotein antigens, it is also important to "detoxify" the protein that is to be expressed so that it will not act as an oncogenic transforming agent. It is to such constructs with the aforementioned advantageous properties that the present invention is directed.

SUMMARY OF THE INVENTION

The present inventors have designed and disclose herein an immunotherapeutic strategy that combines antigen-encoding DNA vaccine compositions in which the antigen-encoding DNA is linked to human CRT or a homologue thereof, a domain or fragment thereof, or other functional derivative that promotes processing of the antigen via the MHC class I pathway and enhanced immunogenicity.

The present invention is directed to a nucleic acid molecule that is an expression vector expressable in a eukaryotic cell, and encodes a chimeric or fusion polypeptide, which molecule comprises:

(a) a first nucleic acid sequence, preferably SEQ ID NO:9, encoding a first polypeptide which is calreticulin (CRT), preferably SEQ ID NO: 10, or a biologically active homologue, domain or fragment thereof,

which homologue, domain or fragment (i) forms complexes with peptides *in vitro;* (ii) when expressed in a cell, participates in folding and assembly of nascent glycoproteins, (iii) when expressed in a cell, associates with peptides transported into the endoplasmic reticulum by transporters that are associated with antigen processing, and/or (iv) inhibits angiogenesis;

(b) a second nucleic acid sequence that is linked in frame to the first nucleic acid sequence and that encodes an antigenic polypeptide or peptide that preferably comprises an epitope that binds to a MHC class I protein, and

(c) operably linked thereto, a promoter active in the eukaryotic cell and, optionally, one or more regulatory elements that enhance expression of the nucleic acid in the cell.

The first nucleic acid sequence may encode one or more CRT fragments or domain selected from the group consisting of (a) N-CRT, (b) P-CRT, (c) S-CRT and (d) a biologically active variant of (a), (b) or (c) 5. Preferably, N-CRT is SEQ ID NO: 14), P-CRT is SEQ ID NO: 15, S-CRT is SEQ ID NO: 16) or each of the domains is a homologue of N-CRT, P-CRT or S-CRT. In a preferred embodiment of the above nucleic acid molecule that encodes a CRT domain, the first nucleic acid sequence is SEQ ID NO: 17 or encodes N-CRT (SEQ ID NO: 14). The first nucleic acid sequence may encodes any two or more of N-CRT (SEQ ID NO: 14), P-CRT (SEQ ID NO: 15), C-CRT (SEQ ID NO:16)or any combination thereof.

In the above nucleic acid molecule, the antigen is preferably one which is present on, or cross-reactive with an epitope of, a pathogenic organism, cell, or virus, most preferably the virus is a human papilloma virus such as HPV- 16 and the antigen is (a) an E7 polypeptide of HPV having the sequence SEQ ID NO:3; (b) an E6 polypeptide of HPV having the sequence SEQ ID NO:6; (c) an in-frame linked combination of E6 and E7 in either orientation; (d) an antigenic fragment of E6 or E7; (e) a non-oncogenic mutant or variant of E6 or E7; or (f) an in-frame linked non-oncogenic mutant/variant E7-E6 combination in either orientation. In a preferred embodiment of the nucleic acid molecule, the antigen is a non-oncogenic mutant or variant of the E7 polypeptide.

A preferred mutant/variant of the E7 polypeptide differs from SEQ ID NO:3 by one or more of the following substitutions: (a) Cys at position 24 to GIy or Ala; (b) GIu at position 26 to GIy or Ala; and (c) Cys at position 9 1 to GIy or Ala, most preferably a polypeptide with the sequence SEQ ID NO:4.

A preferred mutant E6 polypeptide has the sequence SEQ ID NO:6. A preferred non-oncogenic mutant/variant of the E6 differs from SEQ ID NO:6 by one or more of the following substitutions: (a) Cys at position 63 to GIy or Ala; (b) Cys at position 106 to GIy or Ala; and (c) He at position 128 to Thr, most preferably a polypeptide with the sequence SEQ ID NO:7.

The nucleic acid molecule as described above is preferably part of a plasmid, most preferably of the plasmid pNGV4a. In a most preferred embodiment, the nucleic acid is characterized as the plasmid pNGVL4a/CRT/E7(detox), and has the sequence SEQ ID NO:20.

Also provided is a pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) a composition comprising the above nucleic acid molecule.

The invention is also directed to a method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the above

pharmaceutical composition, thereby inducing or enhancing the response. The immune response is preferably one that is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

Also provided is a method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to the subject an effective amount of the above pharmaceutical composition, wherein the second nucleic acid sequence encodes one or more epitopes of E7 or E6, respectively, thereby inhibiting the growth or preventing the re-growth.

In all of the above methods, the administering may be by intramuscular injection, by gene gun intradermal administration, or by needle-free jet injection.

In all of the above methods, the subject is preferably a human and the administering is by a intramuscular injection.

Also provided herein is (1) use of the above nucleic acid molecule, for the manufacture of a medicament for inducing or enhancing an antigen specific immune response in a subject, and (2) use of the above nucleic acid molecule for the manufacture of a medicament for inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1 &2. Flow cytometry analysis **of IFN-γ-secreting E7-specific CD8+ T cell precursors in mice vaccinated with various recombinant DNA vaccines.** Mice (4/group) were immunized with pcDNA3-CRT/E7, pcDNA3-E7/H8P70, pcDNA3-ETA/E7, pcDNA3-Sig/E7/LAMP-l, pcDNA3- E7, and pcDNA3 as described in the Examples. Spleen cells from vaccinated mice were harvested 7 days after a booster vaccination, cultured *in vitro* with MHC class I-restricted E7(aa 49-57) peptide overnight, and stained for both CD8 and intracellular IFN-γ. **Fig.** 1 shows representative flow cytometry data. **Fig.** 2 is a bar graph depicting the number of antigen-specific IFN-γ-secreting CDS^+ T cell precursors/3xl0 ^{5} spleen cells (mean±SD). These results are from one representative experiment of two performed.

Figure 3. *In vivo* tumor treatment experiments to compare the antitumor effect generated by **various DNA vaccine constructs in mice.** Mice (5/ group) were challenged with 10^4 TC-I tumor cells and immunized with various DNA constructs seven days later. Results are expressed as the mean number of lung nodules; *bars,* ± SD. These results are from one representative experiment of two performed.

Figures 4 & 5. Flow cytometric **analysis of** IFN-γ-secreting **E7-specific CD8+ T cell precursors generated** by **various DNA vaccine constructs in vaccinated mice eight weeks after initial vaccination.** Mice (4/group) were immunized with the DNA constructs as described for Fig. 1. Spleen cells from vaccinated mice were harvested 8 weeks after the initial vaccination, cultured *in vitro*

with MHC class I-restricted E7(aa 49-57) peptide overnight, and stained for both CD8 and intracellular EFN-γ. **Fig.** 4 shows representative flow cytometry results. **Fig.** 5 is a graph depicting the number of antigen-specific IFN-γ-secreting $CD8^+$ T cell precursors/3 x1 O^5 spleen cells (mean+SD). These results are from one representative experiment of two performed.

Figure 6. Long-term *in vivo* tumor protection experiments to compare the antitumor effect generated by **various DNA vaccine constructs in vaccinated mice eight weeks after initial vaccination.** Mice (5/group) were immunized and challenged with 10^5 cells/mouse TC-I tumor cells as described in the Examples. Results are expressed as the mean number of lung nodules; *bars,* ± SD. These results are from one representative experiment of two performed.

Figure 7-9. **MHC class I expression of TC-I P3 (A15) and *in vivo* tumor protection experiment using TC-I P3 (A15) tumor cells. Fig.** 7 shows results of flow cytometric analysis characterizing MHC class I expression on TC-I PO and the TC-I P3 (Al 5) subclone. B16 was used as a negative control (dotted line). TC-I PO cells are MHC class I positive (thick line), whereas TC-I P3 (Al 5) exhibits down-regulated MHC class I expression (filled region). **Fig.** 8 shows results of an *in vivo* tumor protection experiments using TC-I P3 (Al 5) tumor cells. Mice (5/group) were vaccinated with 2μg of pcDNA3-E7, pcDNA3-CRT, pcDNA3-CRT/E7 DNA or pcDNA3 empty plasmid. One week after the last vaccination, mice were challenged with $5x10^4$ TC-I P3 (Al 5) tumor cells by subcutaneous injection in the right leg. Mice vaccinated with CRT/E7 DNA provided 100% protection against TC-I P3 (Al 5) when compared to mice vaccinated with other DNA Vaccines (one-way ANOVA, /KO.01). **Fig.** 9 shows results of *in vivo* tumor protection experiments using IFN-γ KO mice. Wild type C57BL/6 mice and IFNγ-depleted C57BL/6 mice (5/group) were vaccinated with 2μg of pcDNA3-CRT/E7 DNA. One week after the last vaccination, mice were challenged with $5xlO^4$ TC-I P3 (Al 5) tumor cells by subcutaneous injection in the right leg as described above. 100% of wt C57BL/6 mice and only 20% of IFNγ-depleted C57BL/6 mice remained free of tumors.

Figure 10 & 11. Intracellular cytokine **staining with flow** cytometry **to demonstrate the number of E7-specific CD8+ T cell precursors in mice vaccinated with** pNGVL4a-CRT/E7(detox). One group of C57BL/6 mice was immunized intramuscularly (i.m.) with 50 μg of DNA vaccine and received a booster with the same regimen one week later. Another group was immunized intradermally (i.d.) via gene gun with 2 μg of DNA vaccine and received a booster with the same regimen one week later. Spleen cells were collected one week after the last vaccination. The number of E7-specific IFN-γ-secreting CDS^+ T cell precursors was analyzed using ICCS followed by flow cytometry. **Fig.** 10 shows representative flow cytometry results (one representative experiment of two performed). **Fig.** 11 shows the results in the form of a bar graph showing the number of interferon-secreting T cells/3xl θ^5 spleen cells.

Figure 12 is a schematic diagram of the **pNGVL4a/CRT/E7(detox)** plasmid vector used for anti-tumor vaccination. Indicated are various cloning sites, promoters and coding sequences.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Partial List of Abbreviations used

APC, antigen presenting cell; CRT, calreticulin; CTL, cytotoxic T lymphocyte; DC, dendritic cell; ECD, extracellular domain; E6, HPV oncoprotein E6; E7, HPV oncoproteinE7; ELISA, enzyme-linked immunosorbent assay; HPV, human papillomavirus; HSP, heat shock protein; Hsp70, mycobacterial heat shock protein 70; IFNγ, interferon-γ; i.m., intramuscular(ly); i.v., intravenous(ly); MHC, major histocompatibility complex; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; β-gal, β-galactosidase

The present inventors and their colleagues have shown that the linkage of CRT and homologues thereof to human papillomavirus type 16 (HPV-16) oncoprotein E7 dramatically increased E7-specific CD8+ T cell precursors and enhance anti-tumor effects against an E7-expressing tumor in vaccinated mice. This is shown in the Examples below, as well as in other publications by the inventors and their colleagues *(e.g.,* Wu & Hung, WO00212281A1 (pub'd 14-FEB-2002); Peng S *et al, J Virol.* 2004 75:8468-76, 2004; Kim JW *et al, Gene Ther.* 77:1011-8, 2004; Peng S *et al, Gene Ther.* 2005, Sep 22; Epub; Cheng WF *et al, Vaccine* 23:3864-74, 2005).

They have now adopted this strategy for clinical trials in patients with HPV-16 associated high-grade squamous intraepithelial lesion of the cervix and in patients with advanced HPV-associated head and neck squamous cell carcinoma. To do so, a CRT/E7 DNA vaccine of a grade consistent with "good manufacturing practice (GMP) requirements was produced in the form of a naked DNA preparation based on the pNGVL4a plasmid developed by the National Gene Vector Lab that has been approved for use in humans. The DNA molecule of the present invention has been termed "pNGVL4a/CRT/E7(detox))". A similar DNA vaccine for the E6 protein is also described below and is referred to as "pNGVL4a/CRT/E6(detox)'\

The pNGVL4a/CRT/E7(detox) DNA vaccine (see below) was generated using the pNGVL4a vector as a backbone. This vector was originally derived from the pNGVL3 vector, which has been approved for human vaccine trials. The pNGVL4a vector includes two immunostimulatory sequences (tandem repeats of CpG dinucleotides) in the noncoding region. Whereas any other plasmid DNA that can transform either APCs or other cells which, via cross-priming, transfer the antigenic moiety to APCs, is useful in the present invention, pNGVLA4 is preferred because it has already been approved for human therapeutic use.

Intramuscular immunization with a secreted form of an antigen or, as here, a DNA vaccine encoding the antigen together with CRT will generate stronger CTL responses than i.m. immunization with a "cytoplasmic" form of antigen, suggesting that the priming of CTL responses after i.m. DNA

immunization is facilitated by the cross-presentation of antigen by non-transfected professional APCs that have acquired the immunogen/antigen indirectly. See, for example, Boyle, JS *et al., Int Immunol* 1997, 9:1897-1906.

In addition, when an oncoprotein or an epitope thereof is the immunizing moiety, it is preferred to reduce the tumorigenic risk of the vaccine itself. The key target antigen, HPV E7 and E6 are oncogenic. In the preferred embodiments herein the DNA encoding the E7 protein was doubly mutated to a form known as "E7(detox) "by substituting nucleotides resulting in substitution of two amino acids at positions $C^{24}G$ (Cys-»Gly) and $E^{26}G$ (Glu→Gly) as described in detail below. These substitutions completely eliminate the capacity of the E7 to binding capacity to Rb, as well as transforming activity. Other substitutions that could achieve the same effect can be readily made by those skilled in the art.

Whereas the present exemplification focused on E7, another embodiment of the present invention comprises an antigenic epitope of the HPV E6 protein, preferably from HPV-16. The E6 proteins from cervical cancer-associated HPV types such as HPV-16 induce proteolysis of the p53 tumor suppressor protein through interaction with E6-AP. Human mammary epithelial cells (MECs) immortalized by E6 display low levels of p53. HPV-16 E6 as well as other cancer-related papillomavirus E6 proteins also binds the cellular protein E6BP (ERC-55). Several different E6 mutations are discussed below after presentation of the "wild type" sequence. Studies describing these mutants (which are incorporated by reference in their entirety) are also discussed in that section.

The present invention also includes the use of a tandem E6-E7 vaccine, using one or more of the mutations described herein to render the oncoproteins inactive with respect to their oncogenic potential *in vivo.* MC Cassetti *et al. {Vaccine 22:520-7,* 2004) described Venezuelan equine encephalitis virus replicon particle (VRP) vaccines encoding the HPV 16 E6 and E7 genes in which the E6 and E7 genes were fused to create one open reading frame and mutated at four or at five amino acid positions (see below). Thus, the present constructs may include one or more epitopes of E6 and E7, which may be arranged in their native order, resulting in either a E6-E7 or E7-E6 sequence, or shuffled in any way that permits the expressed protein to bear the E6 and E7 antigenic epitope in an immunogenic form and result in immunization of the vaccinated recipient. DNA encoding an amino acid spacers between E6 and E7 or between individual epitopes of these proteins may be introduced into the vector, provided again, that the spacers permit the expression and presentation of the epitopes in an immunogenic manner after they have been expressed by transduced host cells.

The orientation in which the two (or more) component polypeptides of the fusion protein are arranged, and therefore, the order of the encoding nucleic acid fragments in the nucleic acid vector, can in certain cases be altered without affecting immunogenicity of the fusion polypeptides proteins and the efficacy of the DNA vaccine, though one orientation may give better results than another. For example,

the HSP70-encoding DNA sequences may be located 5' or 3' to the target antigen-encoding sequences, though E7 (or E6)-HSP70 orientation (N- to C-terminal) is preferred. With CRT, as disclosed herein, the CRT-E7 (or E6) N- to C-terminal orientation is preferred. One skilled in the art can routinely test and compare alternative orientations using methods described herein and those conventional in the art, without undue experimentation.

In a preferred embodiment, these IPP -encoding nucleic acid domains are fused in-frame with the antigen DNA so that the chimeric DNA construct encodes a recombinant fusion polypeptide with the antigen N- (or C-) terminal to the IPP. Of course, if a signal peptide, is included, it should be at the N-terminus of a nascent protein.

As has been disclosed in the inventors' patent and other publications and as will be appreciated by those skilled in the art, the present DNA construct encodes a recombinant polypeptide encoding any MHC class I restricted antigen, exemplified herein by mutant (detox) E7.

SEQUENCES OF POLYPEPTIDES AND NUCLEIC ACIDS

PIasmid and Vector Sequences

The wild-type HPV E7 sequence (nucleotide sequence is SEQ ID NO: 1 used in the present invention, albeit with several mutations, and the wild-type amino acid sequence is SEQ ID NO:2) is shown below. Underlined codons and amino acids are those which are preferably mutated in the present constructs.

```
1/1                                                                  31/11
atg cat gqa gat aca cct aca ttg cat gaa tat atg tta gat ttg caa cca gag aca act
Met His Gl'y Asp Thr Pro Thr Leu His Gl u Tyr Met Leu Asp Leu Gin Pro Gl u Thr Thr
61/21                                                                91/31
gat etc tac tqt tat gag. caa tta aat gac age tea gag gag gag gat gaa ata gat ggt
Asp Leu Tyr Cys Tyr Giu Gin Leu Asn Asp ser Ser Glu Glu Glu Asp Glu lie Asp GIy
121/41                                                               151/51
cca get gqa caa gca gaa ccg gac aga gee cat tac aat att gta ace ttt tgt tgc aag
Pro Ala Gly Gin Ala Glu Pro Asp Arg Ala His Tyr Asn lie val Thr Phe cys cys Lys
181/61                                                               211/71
tgt gac tct acg ctt egg ttg tgc gta caa age aca cac gta gac att cgt act ttg gaa
cys Asp Ser Thr Leu Arg Leu cys val Gin Ser Thr His VaI Asp lie Arg Thr Leu Glu
241/81                                                               271/91
gac ctg tta atg ggc aca eta gqa att gtg tgc ccc ate tgt tct cag gat aag ctt
Asp Leu Leu Met Gly Thr Leu Gly lie VaT cys Pro lie Cys Ser Gin Asp Lys Leu
```

The above sequence differs from the GENEBANK Accession Number NC_001 526 for the E 7 protein which is:

```
MHGDTPTLHE   YMLDLQPETT   DLYCYEQLND   SSEEEDEIDG   PAGQAEPDRA   HYNIVTFCCK
CDSTLRLCVQ   STHVDIRTLE   DLLMGTLGIV   CPICSQKP     9 7  (SEQIDNO:3)
```

The HPV 16 E7 protein binds to its target, Rb, through an L- X-C-X-E motif. Mutations at positions Cys24 and Glu26 of this motif destroy Rb binding and degradation. In addition to these two point mutations in E7, a mutation at a third amino acid, Cys91, destroys the single zinc finger in E7. In a preferred embodiment, all wild type amino acids are mutated to GIy. In another embodiment, these

residues are mutated to Ala. In fact, they can be mutated to any residue that will permit the protein to be expressed in transduced cells, secreted in immunogenic form, taken up by professional APCs, and presented to T cells in a way that will preserve antigenic specificity, while at the same time preventing or lowering the probability that the protein will have oncogenic transforming capacity. The above statement is true with respect to the HPV E6 protein described below.

To reduce oncogenic potential of E7 in a construct of this invention, one or more of the following positions of E7 is mutated:

Original residue	Mutant residue	Preferred codon mutation	Position in SEQ ID NO:3
Cys	Gly (or Ala)	TGT→GGT	24
Glu	Gly (or Ala)	GAG→GGG (or GCG)	26
Cys	Gly (or Ala)	TGC→GGC	91

The E7 (detox) mutant amino acid sequence (SEQ ID NO:4, below) encoded by the preferred vaccine vector has the mutations shown above - namely - a tgt →ggt mutation resulting in a Cys→Gly substitution at position 24 and a gag→ggg mutation resulting in a Glu→Gly substitution at position 26 of SEQ DDNO:2.

```
MHGDTPTLHE  YMLDLQPETT   DLYGYGQLND  SSEEEDEIDG   PAGQAEPDRA  HYNIVTFCCK
CDSTLRLCVQ  STHVDIRTLE   DLLMGTLGIV   CPICSQKP    97   (SEQBDNO:4)
```

E 6 Protein

The wild type HPV E6 amino acid sequence (GENEBANK Accession Number NC_00 1526) (SEQ ID NO:5) is shown below. This sequence has 158 amino acids.

```
MHQKRTAMFQ  DPQERPRKLP  QLCTELQTTI   HDIILECVYC   KQQLLRREVY  DFAFRDLCIV
YRDGNPYAVC_ DKCLKFYSKI  SEYRHYCYSL   YGTTLEQQYN  KPLCDLLIRC   INCQKPLCPE
EKQRHLDKKQ  RFHNIRGRWT  GRCMSCCRSS   RTRRETQL      158
```

The preferred amino acid residues to be mutated are underscored above. The studies of E6 mutants discussed below are based upon a shorter E6, the coding sequence of which encodes 151 amino acids, the N-terminal residue of which was counted as Met-8 in SEQ ID NO:5. That shorter version of E6 is shown below as SEQ ID NO:6.

```
MFQDPQERPR  KLPQLCTELQ  TTIHDIILEC   VYCKQQLLRR  EVYDFAFRDL   CIVYRDGNPY
AVCDKCLKFY  SKISEYRHYC  YSLYGTTLEQ   QYNKPLCDLL  IRCINCQKPL   CPEEKQRHLD
KKQRFHNIRG  RWTGRCMSCC  RSSRTRRETQ   L       151
```

Again, the preferred amino acid residues to be mutated are underscored.

Any nucleotide sequence that encodes this E6 polypeptide, or preferably, one of the mutants thereof discussed below, or an antigenic fragment or epitope thereof, can be used in the present vectors. Codons may be selected by typical codon usage rules.. Other mutations can be

tested and used in accordance with the methods described herein, or those described by Cassetti *et ah, supra.*

To reduce oncogenic potential of E6 in a construct of this invention, one or more of the following positions of E6 is mutated:

Original residue	Mutant residue	Position in SEQ ID NO:5	Position in SEQ ID NO:6
Ile	Thr	135	128
Cys	Gly (or Ala)	70	63
Cys	Gly (or Ala)	113	106

A form of E6(detox) having these three mutations is shown below as SEQ ID NO:7

```
MFQDPQERPR KLPQLCTELQ TTIHDIILEC VYCKQQLLRR EVYDFAFRDL CIVYRDGNPY
AVGDKCLKFY SKISEYRHYC YSLYGTTLEQ QYNKPLCDLL IRCINGQKPL CPEEKQRHLD
KKQRFHNTRG RWTGRCMSCC RSSRTRRETQ L      151
```

These mutations can be achieved using any appropriate coding sequences by mutation of the coding DNA.

The studies describing these mutants (which are incorporated by reference in their entirety) are discussed below. M. Nguyen *et al.* (*J Virol.* <5:13039-48, 2002) described a mutant of HPV-16 E6 deficient in binding α-helix partners which displays reduced oncogenic potential *in vivo.* This mutant, that involves a replacement of He with Thr as position 128 (of SEQ ID NO:6) , may be used in accordance with the present invention to make an E6 DNA vaccine that has a lower risk of being oncogenic. This E6($I^{128}T$) mutant is defective for binding at least a subset of the α-helix partners, including E6AP, the ubiquitin ligase that mediates E6-dependent degradation of the p53 protein,

Cassetti *et al, supra,* examined the effects of mutations four or five amino acid positions in E6 and E7 to inactivate their oncogenic potential. The following mutations were examined (positions based on SEQ ID NO:3 or 5): Eo-$C^{63}G$; E6 $C^{106}G$; E7-$C^{24}G$, E7-$E^{26}G$, and E7 $C^{91}G$. Vaccines encoding mutant or wild type E6 and E7 proteins elicited comparable CTL responses and generated comparable antitumor responses in several HPV 16 $E6^{+}E7^{+}$ tumor challenge models: protection from either C3 or TC-I tumor challenge was observed in 100% of vaccinated mice. Eradication of C3 tumors was observed in approximately 90%. The predicted inactivation of E6 and E7 oncogenic potential was confirmed by demonstrating normal levels of both p53 and Rb proteins in human mammary epithelial cells infected with VRP expressing mutant E6 and E7 genes.

Approaches for Mutagenesis of E6 and E7

The HPV 16 E6 protein contains two zinc fingers important for structure and function; one cysteine (C) amino acid position in each pair of C-X-X-C (where X is any amino acid) zinc finger

motifs are preferably was mutated at E6 positions 63 and 106 (based on SEQ ID NO:5). Mutants are created, for example, using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). HPV16 E6 containing a single point mutation at CyslO6 (of Cys 113 per SEQ IDlSfO:5). Cys neither binds nor facilitates degradation of p53 and is incapable of immortalizing human mammary epithelial cells (MEC), a phenotype dependent upon p53 degradation. A single amino acid substitution at position Cys63 of SEQ ID NO:6 (=Cys70 in SEQ **ID** NO:5) destroys several HPVl 6 E6 functions: p53 degradation, E6TP-1 degradation, activation of telomerase, and, consequently, immortalization of primary epithelial cells.

Sequences of DNA Encoding the Immunogenicity-Potentiating Polypeptide CRT and its Homologues and Domains/Fragments

Calreticulin $(CRT)_5$ a well-characterized ~46 kDa protein was described briefly above, as were a number of its biological and biochemical activities. As used herein, "calreticulin" or "CRT" refers to polypeptides and nucleic acids molecules having substantial identity (defined herein) to the exemplary human CRT sequences as described herein or homologues thereof, such as rabbit and rat CRT - well-known in the art. A CRT polypeptide is a polypeptides comprising a sequence identical to or substantially identical (defined herein) to the amino acid sequence of CRT. An exemplary nucleotide and amino acid sequence for a CRT used in the present compositions and methods are presented below. The terms "calreticulin" or "CRT" encompass native proteins as well as recombinantly produced modified proteins that, when fused with an antigen (at the DNA or protein level) promote the induction of induce immune responses and, promote angiogenesis. , including a CTL response. Thus, the terms "calreticulin" or "CRT" encompass homologues and allelic variants of human CRT, including variants of native proteins constructed by *in vitro* techniques, and proteins isolated from natural sources. The CRT polypeptides of the invention, and sequences encoding them, also include fusion proteins comprising non-CRT sequences, particularly MHC class I-binding peptides; and also further comprising other domains, *e.g.,* epitope tags, enzyme cleavage recognition sequences, signal sequences, secretion signals and the like.

The term "endoplasmic reticulum chaperone polypeptide" as used herein means any polypeptide having substantially the same ER chaperone function as the exemplary chaperone proteins CRT, tapasin, ER60 or calnexin. Thus, the term includes all functional fragments or variants or mimics thereof. A polypeptide or peptide can be routinely screened for its activity as an ER chaperone using assays known in the art, such as those set forth in Example 1. While the invention is not limited by any particular mechanism of action, *in vivo* chaperones promote the correct folding and oligomerization of many glycoproteins in the ER, including the assembly of the MHC class I heterotrimeric molecule (heavy (H)

chain, β2m, and peptide). They also retain incompletely assembled MHC class I heterotrimeric complexes in the ER (Hauri, *FEBS Lett.* 476:32-37, 2000).

The sequences of CRT, including human CRT, are well known in the art (McCauliffe, *J. Clin. Invest. 86-332-5,* 1990; Burns, *Nature* 3(57:476-80, 1994; Coppolino, *Int. J. Biochem. Cell Biol.* 30:553-8, 2000). A sequence of the CRT gene that includes the CRT coding sequence, appears as GenBank Accession No. NM 004343 and is SEQ ID NO:8, below. The portion of this sequence that encodes the CRT protein is residues 68-1319 (shown in upper case below). The stop codon ending this sequence is italicized/underscored.

```
1     gtccgtactg  cagagccgct  gccggagggt  cgttttaaag  ggccgcgttg  ccgcccctc
61    ggcccgccAT  GCTGCTATCC  GTGCCGCTGC  TGCTCGGCCT  CCTCGGCCTG  GCCGTCGCCG
121   AGCCCGCCGT  CTACTTCAAG  GAGCAGTTTC  TGGACGGAGA  CGGGTGGACT  TCCCGCTGGA
181   TCGAATCCAA  ACACAAGTCA  GATTTTGGCA  AATTCGTTCT  CAGTTCCGGC  AAGTTCTACG
241   GTGACGAGGA  GAAAGATAAA  GGTTTGCAGA  CAAGCCAGGA  TGCACGCTTT  TATGCTCTGT
301   CGGCCAGTTT  CGAGCCTTTC  AGCAACAAAG  GCCAGACGCT  GGTGGTGCAG  TTCACGGTGA
361   AACATGAGCA  GAACATCGAC  TGTGGGGGCG  GCTATGTGAA  GCTGTTTCCT  AATAGTTTGG
421   ACCAGACAGA  CATGCACGGA  GACTCAGAAT  ACAACATCAT  GTTTGGTCCC  GACATCTGTG
481   GCCCTGGCAC  CAAGAAGGTT  CATGTCATCT  TCAACTACAA  GGGCAAGAAC  GTGCTGATCA
541   ACAAGGACAT  CCGTTGCAAG  GATGATGAGT  TTACACACCT  GTACACACTG  ATTGTGCGGC
601   CAGACAACAC  CTATGAGGTG  AAGATTGACA  ACAGCCAGGT  GGAGTCCGGC  TCCTTGGAAG
661   ACGATTGGGA  CTTCCTGCCA  CCCAAGAAGA  TAAAGGATCC  TGATGCTTCA  AAACCGGAAG
721   ACTGGGATGA  GCGGGCCAAG  ATCGATGATC  CCACAGACTC  CAAGCCTGAG  GACTGGGACA
781   AGCCCGAGCA  TATCCCTGAC  CCTGATGCTA  AGAAGCCCGA  GGACTGGGAT  GAAGAGATGG
841   ACGGAGAGTG  GGAACCCCCA  GTGATTCAGA  ACCCTGAGTA  CAAGGGTGAG  TGGAAGCCCC
901   GGCAGATCGA  CAACCCAGAT  TACAAGGGCA  CTTGGATCCA  CCCAGAAATT  GACAACCCCG
961   AGTATTCTCC  CGATCCCAGT  ATCTATGCCT  ATGATAACTT  TGGCGTGCTG  GGCCTGGACC
1021  TCTGGCAGGT  CAAGTCTGGC  ACCATCTTTG  ACAACTTCCT  CATCACCAAC  GATGAGGCAT
1081  ACGCTGAGGA  GTTTGGCAAC  GAGACGTGGG  GCGTAACAAA  GGCAGCAGAG  AAACAAATGA
1141  AGGACAAACA  GGACGAGGAG  CAGAGGCTTA  AGGAGGAGGA  AGAAGACAAG  AAACGCAAAG
1201  AGGAGGAGGA  GGCAGAGGAC  AAGGAGGATG  ATGAGGACAA  AGATGAGGAT  GAGGAGGATG
1261  AGGAGGACAA  GGAGGAAGAT  GAGGAGGAAG  ATGTCCCCGG  CCAGGCCAAG  GACGAGCTGi
1321  agragaggcct gcctccaggg ctggactgag gcctgagcgc tcctgccgca gagcttgccg
1381  cgccaaataa tgtctctgtg agactcgaga actttcattt ttttccaggc tggttcggat
1441  ttggggtgga ttttggtttt gttcccctcc tccactctcc cccacccct ccccgccctt
1501  ttttttttt ttttaaact ggtattttat cctttgattc tccttcagcc ctcacccctg
1561  gttctcatct ttcttgatca acatcttttc ttgcctctgt gccccttctc tcatctctta
1621  gctcccctcc aacctggggg gcagtggtgt ggagaagcca caggcctgag atttcatctg
1681  ctctccttcc tggagcccag aggagggcag cagaaggggg tggtgtctcc aaccccccag
1741  cactgaggaa gaacggggct cttctcattt caccctccc tttctcccct gcccccagga
1801  ctgggccact tctgggtggg gcagtgggtc ccagattggc tcacactgag aatgtaagaa
1861  ctacaaacaa aatttctatt aaattaaatt ttgtgtctc              1899
```

The human CRT coding sequence is shown below (SEQ ID NO:9):

```
1     atgctgctat  ccgtgccgct  gctgctcggc  ctcctcggcc  tggccgtcgc  cgagcccgcc
61    gtctacttca  aggagcagtt  tctggacgga  gacgggtgga  cttcccgctg  gatcgaatcc
121   aaacacaagt  cagattttgg  caaattcgtt  ctcagttccg  gcaagttcta  cggtgacgag
181   gagaaagata  aaggtttgca  gacaagccag  gatgcacgct  tttatgctct  gtcggccagt
241   ttcgagcctt  tcagcaacaa  aggccagacg  ctggtggtgc  agttcacggt  gaaacatgag
301   cagaacatcg  actgtggggg  cggctatgtg  aagctgtttc  ctaatagttt  ggaccagaca
361   gacatgcacg  gagactcaga  atacaacatc  atgtttggtc  ccgacatctg  tggccctggc
421   accaagaagg  ttcatgtcat  cttcaactac  aagggcaaga  acgtgctgat  caacaaggac
481   atccgttgca  aggatgatga  gtttacacac  ctgtacacac  tgattgtgcg  gccagacaac
541   acctatgagg  tgaagattga  caacagccag  gtggagtccg  gctccttgga  agacgattgg
601   gacttcctgc  cacccaagaa  gataaaggat  cctgatgctt  caaaaccgga  agactgggat
661   gagcgggcca  agatcgatga  tcccacagac  tccaagcctg  aggactggga  caagcccgag
721   catatccctg  accctgatgc  taagaagccc  gaggactggg  atgaagagat  ggacggagag
781   tgggaacccc  cagtgattca  gaaccctgag  tacaagggtg  agtggaagcc  ccggcagatc
841   gacaacccag  attacaaggg  cacttggatc  cacccagaaa  ttgacaaccc  cgagtattct
```

```
901   cccgatccca  gtatctatgc  ctatgataac  tttggcgtgc  tgggcctgga  cctctggcag
961   gtcaagtctg  gcaccatctt  tgacaacttc  ctcatcacca  acgatgaggc  atacgctgag
1021  gagtttggca  acgagacgtg  gggcgtaaca  aaggcagcag  agaaacaaat  gaaggacaaa
1081  caggacgagg  agcagaggct  taaggaggag  gaagaagaca  agaaacgcaa  agaggaggag
1141  gaggcagagg  acaaggagga  tgatgaggac  aaagatgagg  atgaggagga  tgaggaggac
1201  aaggaggaag  atgaggagga  agatgtcccc  ggccaggcca  aggacgagct  ataa  1251
```

The amino acid sequence of the human CRT protein (GenBank Accession No. NM 004343) (SEQ ID NO: 10) is shown below:

```
1    MLLSVPLLLG  LLGLAVAEPA  VYFKEQFLDG  DGWISRWIES  KHKSDFGKFV  LSSGKFYGDE
61   EKDKGLQTSQ  DARFYALSAS  FEPFSNKGQT  LVVQFTVKHE  QNIDCGGGYV  KLFPNSLDQT
121  DMHGDSEYNI  MFGPDICGPG  TKKVHVIFNY  KGKNVLINKD  IRCKDDEFTH  LYTLIVRPDN
181  TYEVKIDNSQ  VESGSLEDDW  DFLPPKKIKD  PDASKPEDWD  ERAKIDDPTD  SKPEDWDKPE
241  HIPDPDAKKP  EDWDEEMDGE  WEPPVIQNPE  YKGEWKPRQI  DNPDYKGTWI  HPEIDNPEYS
301  PDPSIYAYDN  FGVLGLDLWQ  VKSGIIFDNF  LITNDEAYAE  EFGNETWGVT  KAAEKQMKDK
361  QDEEQRLKEE  EEDKKRKEEE  EAEDKEDDED  KDEDEEDEED  KEEDEEEDVP  GQAKDEL  417
```

The amino acid sequence of the rabbit CRT protein (GenBank Accession No. P15253) (SEQ ID NO:11) is shown below

```
1    MLLPVPLLLG  LLGLAAAEPV  VYFKEQFLDG  DGWTERWIES  KHKSDFGKFV  LSSGKFYGDQ
61   EKDKGLQTSQ  DARFYALSAR  FEPFSNKGQP  LVVQFTVKHE  QNIDCGGGYV  KLFPAGLDQK
121  DMHGDSEYNI  MFGPDICGPG  TKKVHVIFNY  KGKNVLINKD  IRCKDDEFTH  LYTLIVRPDN
181  TYEVKIDNSQ  VESGSLEDDW  DFLPPKKIKD  PDASKPEDWD  ERAKIDDPTD  SKPEDWDKPE
241  HIPDPDAKKP  EDWDEEMDGE  WEPPVIQNPE  YKGEWKPRQI  DNPDYKGTWI  HPEIDNPEYS
301  PDANIYAYDS  FAVLGLDLWQ  VKSGTIFDNF  LITNDEAYAE  EFGNETWGVT  KTAEKQMKDK
361  QDEEQRLKEE  EEEKKRKEEE  EAEEDEEDKD  DKEDEDEDEE  DKDEEEEEAA  AGQAKDEL  418
```

The rabbit nucleotide sequence encoding CRT is shown below (SEQ ID NO: 12) (including the stop codon which is underscored and not counted)

```
1     atgctgctcc  ctgtgccgct  gctgctcggc  ctgctcggcc  tggccgccgc  cgagcccgtc
61    gtctacttca  aggagcagtt  tctggacgga  gatgggtgga  ccgagcgctg  gatcgaatcc
121   aaacacaagt  ccgattttgg  caaattcgtc  ctcagttcgg  gcaagttcta  cggcgatcag
181   gagaaagata  aagggctgca  gaccagccag  gacgcccgct  tctacgccct  gtcggcccga
241   ttcgagccgt  tcagcaacaa  gggccagcca  ctggtggtgc  agttcaccgt  gaaacacgag
301   cagaacattg  actgcggggg  cggctacgtg  aagctgtttc  cggccggcct  ggaccagaag
361   gacatgcacg  gggactctga  gtacaacatc  atgtttggtc  ctgacatctg  tggccccggc
421   accaagaagg  ttcacgtcat  cttcaactac  aagggcaaga  acgtgctgat  caacaaggac
481   atccgttgca  aggacgacga  gttcacacac  ctgtacacgc  tgatcgtgcg  gccggacaac
541   acgtatgagg  tgaagattga  caacagccag  gtggagtcgg  gctccctgga  ggatgactgg
601   gacttcctac  cccccaagaa  gataaaggac  ccagatgcct  cgaagcctga  agactgggac
661   gagcgggcca  agatcgacga  ccccacggac  tccaagcccg  aggactggga  caagcccgag
721   cacatccccg  acccggacgc  gaagaagccc  gaagactggg  acgaagaaat  ggacggagag
781   tgggagccgc  cggtgattca  gaaccccgag  tacaagggtg  agtggaagcc  gcggcagatc
841   gacaaccccg  attacaaagg  cacctggatc  caccccgaaa  tcgacaaccc  cgagtactcg
901   cccgacgcta  acatctatgc  ctacgacagc  tttgccgtgc  tgggcttgga  cctctggcag
961   gtcaagtcgg  gcaccatctt  cgacaacttc  ctcatcacca  acgatgaggc  gtacgcagag
1021  gagtttggca  acgagacgtg  gggcgtcacc  aagacggccg  agaagcagat  gaaagacaag
1081  caggacgagg  agcagcggct  gaaggaggag  gaggaggaga  agaagcggaa  ggaggaggag
1141  gaggccgagg  aggacgagga  ggacaaggac  gacaaggagg  acgaggatga  ggacgaggag
1201  gacaaggacg  aggaggagga  ggaggcggcc  gccggccagg  ccaaggacga  gctg  taa  1254
```

Rat CRT protein (GenBank Accession No. NM 022399) (SEQ ID NO: 13) is shown below.

```
1    MLLSVPLLLG  LLGLAAADPA  IYFKEQFLDG  DAWTNRWVES  KHKSDFGKFV  LSSGKFYGDQ
61   EKDKGLQTSQ  DARFYALSAR  FEPFSNKGQT  LVVQFTVKHE  QNIDCGGGYV  KLFPGGLDQK
121  DMHGDSEYNI  MFGPDICGPG  TKKVHVIFNY  KGKNVLINKD  IRCKDDEFTH  LYTLIVRPDN
181  TYEVKIDNSQ  VESGSLEDDW  DFLPPKKIKD  PDAAKPEDWD  ERAKIDDPTD  SKPEDWDKPE
241  HIPDPDAKKP  EDWDEEMDGE  WEPPVIQNPE  YKGEWKPRQI  DNPDYKGTWI  HPEIDNPEYS
```

301 PDANIYAYDS FAVLGLDLWQ VKSGTI FDNF LITNDEAYAE EFGNETWGVT KAAEKQMKDK
361 QDEEQRLKEE EEDKKRKEEE EAEDKEDEDD RDEDEDEEDE KEEDEEDATG QAKDEL 416

The corresponding coding sequence is not shown for the sake of brevity.

Table 1 compares aligned sequences of human, rabbit and rat CRT (SEQ ID NO:1 1, 12, and 13), respectively. The changes from human to rabbit and human to rat are underscored. As stated above, these proteins are highly conserved, and most of the amino acid differences between species are conservative in nature. Most of the variation is found in the alignment of the approximately 36 C-terminal residues. Thus, for the present invention, although human CRT is preferred, DNA encoding any homologue of CRT from any species that has the requisite biological activity (as an IPP) or any active domain or fragment thereof, may be used in place of human CRT or a domain thereof.

Table 1: Sequence Alignment of Human, Rabbit and Rat* CRT Polypeptides

H	1	MLLSVPLLLG	LLGLAVAEPA	VYFKEQFLDG	DGWTSRWIES	KHKSDFGKFV	LSSGKFYGDE	
Rb	1	MLLPVPLLLG	LLGLAAAEPV	VYFKEQFLDG	DGWTERWIES	KHKSDFGKFV	LSSGKFYGDQ	
Rt	1	MLLSVPLLLG	LLGLAAADPA	IYFKEQFLDG	DAWTNRWVES	KHKSDFGKFV	LSSGKFYGDQ	
H	61	EKDKGLQTSQ	DARFYALSAS	FEPFSNKGQT	LVVQFTVKHE	QNIDCGGGYV	KLFPNSLDQT	
Rbi	61	EKDKGLQTSQ	DARFYALSAR_	FEPFSNKGQP	LVVQFTVKHE	QNIDCGGGYV	KLFPAGLDQK	
Rt	61	EKDKGLQTSQ	DARFYALSAR_	FEPFSNKGQT	LVVQFTVKHE	QNIDCGGGYV	KLFPGGLDQK	
H	121	DMHGDSEYNI	MFGPDICGPG	TKKVHVIFNY	KGKNVLINKD	IRCKDDEFTH	LYTLIVRPDN	
Rb121		DMHGDSEYNI	MFGPDICGPG	TKKVHVIFNY	KGKNVLINKD	IRCKDDEFTH	LYTLIVRPDN	
Rt	121	DMHGDSEYNI	MFGPDICGPG	TKKVHVIFNY	KGKNVLINKD	IRCKDDEFTH	LYTLIVRPDN	
H	181	TYEVKIDNSQ	VESGSLEDDW	DFLPPKKIKD	PDASKPEDWD	ERAKIDDPTD	SKPEDWDKPE	
Rb181		TYEVKIDNSQ	VESGSLEDDW	DFLPPKKIKD	PDASKPEDWD	ERAKIDDPTD	SKPEDWDKPE	
Rt	181	TYEVKIDNSQ	VESGSLEDDW	DFLPPKKIKD	PDAAKPEDWD	ERAKIDDPTD	SKPEDWDKPE	
H	241	HIPDPDAKKP	EDWDEEMDGE	WEPPVIQNPE	YKGEWKPRQI	DNPDYKGTWI	HPEIDNPEYS	
Rb241		HIPDPDAKKP	EDWDEEMDGE	WEPPVIQNPE	YKGEWKPRQI	DNPDYKGTWI	HPEIDNPEYS	
Rt	241	HIPDPDAKKP	EDWDEEMDGE	WEPPVIQNPE	YKGEWKPRQI	DNPDYKGTWI	HPEIDNPEYS	
H	301	PDPSIYAYDN	FGVLGLDLWQ	VKSGTIFDNF	LITNDEAYAE	EFGNETWGVT	KAAEKQMKDK	
Rb301		PDANIYAYDS_	FAVLGLDLWQ	VKSGTIFDNF	LITNDEAYAE	EFGNETWGVT	KTAEKQMKDK	
Rt	301	PDANIYAYDS_	FAVLGLDLWQ	VKSGTIFDNF	LITNDEAYAE	EFGNETWGVT	KAAEKQMKDK	
H	361	QDEEQRLKEE	EEDKKRKEEE	EAEDKEDDED	K DEDEEDEED	KEEDEEEDVPG	QAKDEL	417
Rb361		QDEEQRLKEE	EEEKKRKEEE	EAEEDEEDKDDKEDEDEJDEED		KDEEEEEAAAG	QAKDEL	418
Rt	361	QDEEQRLKEE	EEDKKRKEEE	EAEDKEDEDD	R_DEDE_DEEDE	KEEDEEJDATG	QAKDEL	416

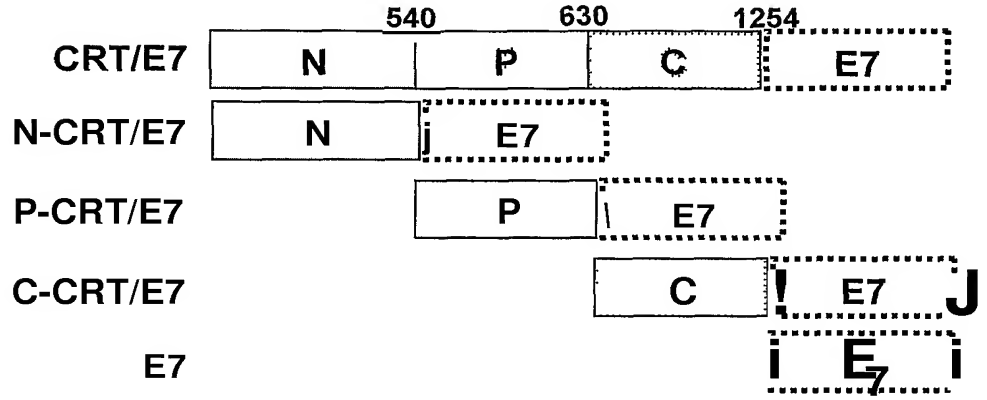
*H: human; Rb: rabbit; Rt: rat

CRT Domains

The present inventors and colleagues (Cheng *et al, supra;* incorporated by reference in its entirety) that DNA vaccines encoding each of the N, P, and C domains of CRT chimerically linked to HPY-16 E7 elicited potent antigen-specific CD8+ T cell responses and antitumor immunity in mice vaccinated i.d., by gene gun administration. N-CRT/E7, P-CRT/E7 or C-CRT/E7 DNA each exhibited

significantly increased numbers of E7-specific $CD8^+$ T cell precursors and impressive antitumor effects against E7-expressing tumors when compared with mice vaccinated with E7 DNA (antigen only). N-CRT DNA administration also resulted in anti-angiogenic antitumor effects. Thus, cancer therapy using DNE encoding N-CRT linked to a tumor antigen may be used for treating tumors through a combination of antigen-specific immunotherapy and inhibition of angiogenesis.

The constructs comprising CRT or one of its domains linked to E7 is illustrated schematically below.



The amino acid sequences of the 3 human CRT domains are shown as annotations of the full length protein (SEQ ID NO:10). The N domain comprises residues 1-170 (normal text); the P domain comprises residues 171-269 (underscored); and the C domain comprises residues 270-417 (bold/italic)

```
1   MLLSVPLLLG LLGLAVAEPA VYFKEQFLDG DGWTSRWIES KHKSDFGKFV LSSGKFYGDE
61  EKDKGLQTSQ DARFYALSAS FEPFSNKGQT LVVQFTVKHE QNIDCGGGYV KLFPNSLDQT
121 DMHGDSEYNI MFGPDICGPG TKKVHVIFNY KGKNVLINKD IRCKDDEFTH LYTLIVRPDN
181 TYEVKIDNSQ VESGSLEDDW DFLPPKKIKD PDASKPEDWD ERAKIDDPTD SKPEDWDKPE
241 HIPDPDAKKP EDWDEEMDGE WEPPVIQNPE YKGEWKPRQJ DNPDYKGTWI HPEIDNPEYS
301 PDPSIYAYDN FGVLGLDLWQ VKSGTIFDNF LITNDEAYAE EFGNETWGVT KAAEKQMKDK
361 QDEEQRLKEE EEDKKRKEEE EAEDKEDDED KDEDEEDEED KEEDEEEDVP GQAKDEL 417
```

The sequences of the three domains are shown as separate polypeptides below:

Human N-CRT (SEQ ID NO: 14)

```
1   MLLSVPLLLG LLGLAVAEPA VYFKEQFLDG DGWTSRWIES KHKSDFGKFV LSSGKFYGDE
61  EKDKGLQTSQ DARFYALSAS FEPFSNKGQT LVVQFTVKHE QNIDCGGGYV KLFPNSLDQT
121 DMHGDSEYNI MFGPDICGPG TKKVHVIFNY KGKNVLINKD IRCKDDEFTH      170
```

Human P-CRT (SEQ ID NO: 15)

```
1   LYTLIVRPDN TYEVKIDNSQ VESGSLEDDW DFLPPKKIKD PDASKPEDWD ERAKIDDPTD
61  SKPEDWDKPE HIPDPDAKKP EDWDEEMDGE WEPPVIQNPE YKGEWKPRQ       109
```

Human C-CRT (SEQ ID NO: 16)

```
1   IDNPDYKGTW IHPEIDNPEY SPDPSIYAYD NFGVLGLDLW QVKSGTIFDN FLITNDEAYA
61  EEFGNETWGV TKAAEKQMKD KQDEEQRLKE EEEDKKRKEE EEAEDKEDDE DKDEDEEDEE
121 DKEEDEEEDV PGQAKDEL                                    138
```

The present vectors may comprises DNA encoding one or more of these domain sequences, which are shown by annotation of SEQ ID NO:9, below, wherein the N-domain sequence is upper case, the P-domain sequence is lower case/italic/underscored, and the C domain sequence is lower case. The stop codon is also shown but not counted.

1	ATGCTGCTAT	CCGTGCCGCT	GCTGCTCGGC	CTCCTCGGCC	TGGCCGTCGC	CGAGCCCGCC
61	GTCTACTTCA	AGGAGCAGTT	TCTGGACGG A	GACGGGTGGA	CTTCCCGCTG	GATCGAATCC
121	AAACACAAGT	CAGATTTTGG	CAAATTCGTT	CTCAGTTCCG	GCAAGTTCTA	CGGTGACGAG
181	GAGAAAGATA	AAGGTTTGCA	GACAAGCCAG	GATGCACGCT	TTTATGCTCT	GTCGGCCAGT
241	TTCGAGCCTT	TCAGCAACAA	AGGCCAGACG	CTGGTGGTGC	AGTTCACGGT	GAAACATGAG
301	CAGAACATCG	ACTGTGGGGG	CGGCTATGTG	AAGCTGTTTC	CTAATAGTTT	GGACCAGACA
361	GACATGCACG	GAGACTCAGA	ATACAACATC	ATGTTTGGTC	CCGACATCTG	TGGCCCTGGC
421	ACCAAGAAGG	TTCATGTCAT	CTTCAACTAC	AAGGGCAAGA	ACGTGCTGAT	CAACAAGGAC
481	ATCCGTTGCA	AGGATGATGA	GTTTACACAC	CTGTACACAC	TGATTGTGCG	GCCAGACAAC
541	*acctataaaa*	*taaaaattaa*	*caacaccaα*	*αtααaαtccα*	*αctcctt ααa*	*aαacαattpQ*
601	*αacttcct αc*	*cacccaa αaa*	*αataaa ααat*	*cctαatαctt*	*caaaacc ααa*	*agact αααat*
661	*αaαcααqcca*	*aαatcαatαa*	*tcccacaαac*	*tccaaαcctg*	*aααactαααa*	*caactcccαag*
721	*catatccct α*	*accct αatαc*	*taaαaaαccc*	*αaααactααα*	*atαaaαaαat*	*ααacααaαaα*
781	*tααgaacccc*	*caαtαattca*	*oaaccctαaα*	tacaagggtg	agtggaagcc	ccggcagatc
841	gacaacccag	attacaaggg	cacttggatc	cacccagaaa	ttgacaaccc	cgagtattct
901	cccgatccca	gtatctatgc	ctatgataac	tttggcgtgc	tgggcctgga	cctctggcag
961	gtcaagtctg	gcaccatctt	tgacaacttc	ctcatcacca	acgatgaggc	atacgctgag
1021	gagtttggca	acgagacgtg	gggcgtaaca	aaggcagcag	agaaacaaat	gaaggacaaa
1081	caggacgagg	agcagaggct	taaggaggag	gaagaagaca	agaaacgcaa	agaggaggag
1141	gaggcagagg	acaaggagga	tgatgaggac	aaagatgagg	atgaggagga	tgaggaggac
1201	aaggaggaag	atgaggagga	agatgtcccc	ggccaggcca	aggacgagct	gtafl 1251

The coding sequence for each separate domain is provided below:

Human N-CRT DNA (SEQ ID NO: 17)

1	ATGCTGCTAT	CCGTGCCGCT	GCTGCTCGGC	CTCCTCGGCC	TGGCCGTCGC	CGAGCCCGCC
61	GTCTACTTCA	AGGAGCAGTT	TCTGGACGG A	GACGGGTGGA	CTTCCCGCTG	GATCGAATCC
121	AAACACAAGT	CAGATTTTGG	CAAATTCGTT	CTCAGTTCCG	GCAAGTTCTA	CGGTGACGAG
181	GAGAAAGATA	AAGGTTTGCA	GACAAGCCAG	GATGCACGCT	TTTATGCTCT	GTCGGCCAGT
241	TTCGAGCCTT	TCAGCAACAA	AGGCCAGACG	CTGGTGGTGC	AGTTCACGGT	GAAACATGAG
301	CAGAACATCG	ACTGTGGGGG	CGGCTATGTG	AAGCTGTTTC	CTAATAGTTT	GGACCAGACA
361	GACATGCACG	GAGACTCAGA	ATACAACATC	ATGTTTGGTC	CCGACATCTG	TGGCCCTGGC
421	ACCAAGAAGG	TTCATGTCAT	CTTCAACTAC	AAGGGCAAGA	ACGTGCTGAT	CAACAAGGAC
481	ATCCGTTGCA	AGGATGATGA	GTTTACACAC	CTGTACACAC	TGATTGTGCG	GCCAGACAAC

Human P-CRT DNA (SEQ ID NO: 18)

1	acctatgagg	tgaagattga	caacagccag	gtggagtccg	gctccttgga	agacgattgg
61	gacttcctgc	cacccaagaa	gataaaggat	cctgatgctt	caaaaccgga	agactgggat
121	gagcgggcca	agatcgatga	tcccacagac	tccaagcctg	aggactggga	caagcccgag
181	catatccctg	accctgatgc	taagaagccc	gaggactggg	atgaagagat	ggacggagag
241	tgggaacccc	cagtgattca	gaaccct 267			

Human C-CRT DNA (SEQ ID NO: 19)

1	gagtacaagg	gtgagtggaa	gccccggcag	atcgacaacc	cagattacaa	gggcacttgg
61	atccacccag	aaattgacaa	ccccgagtat	tctcccgatc	ccagtatcta	tgcctatgat
121	aactttggcg	tgctgggcct	ggacctctgg	caggtcaagt	ctggcaccat	ctttgacaac
181	ttcctcatca	ccaacgatga	ggcatacgct	gaggagtttg	gcaacgagac	gtggggcgta
241	acaaaggcag	cagagaaaca	aatgaaggac	aaacaggacg	aggagcagag	gcttaaggag
301	gaggaagaag	acaagaaacg	caaagaggag	gaggaggcag	aggacaagga	ggatgatgag
361	gacaaagatg	aggatgagga	ggatgaggag	gacaaggagg	aagatgagga	ggaagatgtc
421	cccggccagg	ccaaggacga	gctg 444			

Alternatively, any nucleotide sequences that encodes these domains may be used in the present constructs. Thus, for use in humans, the sequences may be further codon-optimized.

The present construct may employ combinations of one or more CRT domains, in any of a number of orientations. Using the designations N^{CRT}, P^{CRT} and C^{CRT} io designate the domains, the following are but a few examples of the combinations that may be used in the DNA vaccine vectors of th present invention (where it is understood that Ag can be any antigen, preferably E7(detox) or E6 (detox).

$N^{CRT}\_P^{CRT}\_Ag$;
N^{CRT} - N^{CRT} - N^{CRT} - Ag;
$P^{CRT}\_pCRT\_Ag$;

$N^{CRT}\_pCRT\_Ag$;
$pCRT\_pCRT\_Ag$.
$N^{CRT}\_pCRT\_Ag$;

N^{CRT} - C^{CRT}- Ag;
$pCRT\_QCRT\_Ag$;
etc.

N^{CRT} - N^{CRT} - Ag;
$pCRT\_N^{CRT}\_Ag$.

The present invention may employ shorter fragments of CRT provided such fragments can enhance the immune response to an antigen with which they are paired. Shorter peptides from the CRT or domain sequences shown above that have the ability to promote protein processing via the MHC-I class I pathway are also included, and may be defined by routine experimentation.

A most preferred vector construct of a complete chimeric nucleic acid of the invention, is shown below (SEQ ID NO:20). The sequence is annotated to show plasmid-derived nucleotides (lower case letters), CRT-derived nucleotides (upper case bold letters), and HPV-E7-derived nucleotides (upper case, italicized/underlined letters). Note that 5 plasmid nucleotides are found between the CRT and E7 coding sequences and that the stop codon for the E7 sequence is double underscored. This plasmid is also referred to as pNGVL4a-CRT/E7(detox).

```
1     gctccgcccc cctgacgagc atcacaaaaa tcgacgctca agtcagaggt ggcgaaaccc
61    gacaggacta taaagatacc aggcgtttcc ccctggaagc tccctcgtgc gctctcctgt
121   tccgaccctg ccgcttaccg gatacctgtc cgcctttctc ccttcgggaa gcgtggcgct
181   ttctcatagc tcacgctgta ggtatctcag ttcggtgtag gtcgttcgct ccaagctggg
241   ctgtgtgcac gaaccccccg ttcagcccga ccgctgcgcc ttatccggta actatcgtct
301   tgagtccaac ccggtaagac acgacttatc gccactggca gcagccactg gtaacaggat
361   tagcagagcg aggtatgtag gcggtgctac agagttcttg aagtggtggc ctaactacgg
421   ctacactaga agaacagtat ttggtatctg cgctctgctg aagccagtta ccttcggaaa
481   aagagttggt agctcttgat ccggcaaaca aaccaccgct ggtagcggtg gtttttttgt
541   ttgcaagcag cagattacgc gcagaaaaaa aggatctcaa gaagatcctt tgatcttttc
601   tacggggtct gacgctcagt ggaacgaaaa ctcacgttaa gggattttgg tcatgagatt
661   atcaaaaagg atcttcacct agatcctttt aaattaaaaa tgaagtttta aatcaatcta
721   aagtatatat gagtaaactt ggtctgacag ttaccaatgc ttaatcagtg aggcacctat
781   ctcagcgatc tgtctatttc gttcatccat agttgcctga ctcgggggggg gggggcgctg
841   aggtctgcct cgtgaagaag gtgttgctga ctcataccag ggcaacgttg ttgccattgc
901   tacaggcatc gtggtgtcac gctcgtcgtt tggtatggct tcattcagct ccggttccca
961   acgatcaagg cgagttacat gatccccat gttgtgcaaa aaagcggtta gctccttcgg
1021  tcctccgatc gttgtcagaa gtaagttggc cgcagtgtta tcactcatgg ttatggcagc
1081  actgcataat tctcttactg tcatgccatc cgtaagatgc ttttctgtga ctggtgagta
1141  ctcaaccaag tcattctgag aatagtgtat gcggcgaccg agttgctctt gcccggcgtc
1201  aatacgggat aataccgcgc cacatagcag aactttaaaa gtgctcatca ttggaaaacg
1261  ttcttcgggg cgaaaactct caaggatctt accgctgttg agatccagtt cgatgtaacc
1321  cactcgtgca cctgaatcgc cccatcatcc agccagaaag tgagggagcc acggttgatg
1381  agagctttgt tgtaggtgga ccagttggtg attttgaact tttgctttgc cacggaacgg
1441  tctgcgttgt cgggaagatg cgtgatctga tccttcaact cagcaaaagt tcgatttatt
1501  caacaaagcc gccgtcccgt caagtcagcg taatgctctg ccagtgttac aaccaattaa
1561  ccaattctga ttagaaaaac tcatcgagca tcaaatgaaa ctgcaattta ttcatatcag
1621  gattatcaat accatatttt tgaaaaagcc gtttctgtaa tgaaggagaa aactcaccga
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1681  ggcagttcca taggatggca agatcctggt atcggtctgc gattccgact cgtccaacat
1741  caatacaacc tattaatttc ccctcgtcaa aaataaggtt atcaagtgag aaatcaccat
1801  gagtgacgac tgaatccggt gagaatggca aaagcttatg catttctttc cagacttgtt
1861  caacaggcca gccattacgc tcgtcatcaa aatcactcgc atcaaccaaa ccgttattca
1921  ttcgtgattg cgcctgagcg agacgaaata cgcgatcgct gttaaaagga caattacaaa
1981  caggaatcga atgcaaccgg cgcaggaaca ctgccagcgc atcaacaata ttttcacctg
2041  aatcaggata ttcttctaat acctggaatg ctgttttccc ggggatcgca gtggtgagta
2101  accatgcatc atcaggagta cggataaaat gcttgatggt cggaagaggc ataaattccg
2161  tcagccagtt tagtctgacc atctcatctg taacatcatt ggcaacgcta cctttgccat
2221  gtttcagaaa caactctggc gcatcgggct tcccatacaa tcgatagatt gtcgcacctg
2281  attgcccgac attatcgcga gcccatttat acccatataa atcagcatcc atgttggaat
2341  ttaatcgcgg cctcgagcaa gacgtttccc gttgaatatg gctcataaca ccccttgtat
2401  tactgtttat gtaagcagac agttttattg ttcatgatga tatatttta tcttgtgcaa
2461  tgtaacatca gagattttga gacacaacgt ggctttcccc ccccccccat tattgaagca
2521  tttatcaggg ttattgtctc atgagcggat acatatttga atgtatttag aaaaataaac
2581  aaatagggct tccgcgcaca tttccccgaa aagtgccacc tgacgtctaa gaaaccatta
2641  ttatcatgac attaacctat aaaaataggc gtatcacgag gccctttcgt ctcgcgcgtt
2701  tcggtgatga cggtgaaaac ctctgacaca tgcagctccc ggagacggtc acagcttgtc
2761  tgtaagcgga tgccgggagc agacaagccc gtcagggcgc gtcagcgggt gttggcgggt
2821  gtcggggctg gcttaactat gcggcatcag agcagattgt actgagagtg caccatatgc
2881  ggtgtgaaat accgcacaga tgcgtaagga gaaaataccg catcagattg gctattggcc
2941  attgcatacg ttgtatccat atcataatat gtacatttat attggctcat gtccaacatt
3001  accgccatgt tgacattgat tattgactag ttattaatag taatcaatta cggggtcatt
3061  agttcatagc ccatatatgg agttccgcgt tacataactt acggtaaatg gcccgcctgg
3121  ctgaccgccc aacgacccccc gcccattgac gtcaataatg acgtatgttc ccatagtaac
3181  gccaataggg actttccatt gacgtcaatg ggtggagtat ttacggtaaa ctgcccactt
3241  ggcagtacat caagtgtatc atatgccaag tacgcccccct attgacgtca atgacggtaa
3301  atggcccgcc tggcattatg cccagtacat gaccttatgg gactttccta cttggcagta
3361  catctacgta ttagtcatcg ctattaccat ggtgatgcgg ttttggcagt acatcaatgg
3421  gcgtggatag cggtttgact cacggggatt tccaagtctc caccccattg acgtcaatgg
3481  gagtttgttt tggcaccaaa atcaacggga ctttccaaaa tgtcgtaaca actccgcccc
3541  attgacgcaa atgggcggta ggcgtgtacg gtgggaggtc tatataagca gagctcgttt
3601  agtgaaccgt cagatcgcct ggagacgcca tccacgctgt tttgacctcc atagaagaca
3661  ccgggaccga tccagcctcc gcggccggga acggtgcatt ggaacgcgga ttccccgtgc
3721  caagagtgac gtaagtaccg cctatagact ctataggcac acccctttgg ctcttatgca
3781  tgctatactg tttttggctt ggggcctata cacccccgct tccttatgct ataggtgatg
3841  gtatagctta gcctataggt gtgggttatt gaccattatt gaccactcca acggtggagg
3901  gcagtgtagt ctgagcagta ctcgttgctg ccgcgcgcgc caccagacat aatagctgac
3961  agactaacag actgttcctt tccatgggtc ttttctgcag tcaccgtcgt cgacATGCTG
4021  CTATCCGTGC CGCTGCTGCT CGGCCTCCTC GGCCTGGCCG TCGCCGAGCC TGCCGTCTAC
4081  TTCAAGGAGC AGTTTCTGGA CGGGGACGGG TGGACTTCCC GCTGGATCGA ATCCAAACAC
4141  AAGTCAGATT TTGGCAAATT CGTTCTCAGT TCCGGCAAGT TCTACGGTGA CGAGGAGAAA
4201  GATAAAGGTT TGCAGACAAG CCAGGATGCA CGCTTTTATG CTCTGTCGGC CAGTTTCGAG
4261  CCTTTCAGCA ACAAAGGCCA GACGCTGGTG GTGCAGTTCA CGGTGAAACA TGAGCAGAAC
4321  ATCGACTGTG GGGGCGGCTA TGTGAAGCTG TTTCCTAATA GTTTGGACCA GACAGACATG
4381  CACGGAGACT CAGAATACAA CATCATGTTT GGTCCCGACA TCTGTGGCCC TGGCACCAAG
4441  AAGGTTCATG TCATCTTCAA CTACAAGGGC AAGAACGTGC TGATCAACAA GGACATCCGT
4501  TGCAAGGATG ATGAGTTTAC ACACCTGTAC ACACTGATTG TGCGGCCAGA CAACACCTAT
4561  GAGGTGAAGA TTGACAACAG CCAGGTGGAG TCCGGCTCCT TGGAAGACGA TTGGGACTTC
4621  CTGCCACCCA AGAAGATAAA GGATCCTGAT GCTTCAAAAC CGGAAGACTG GGATGAGCGG
4681  GCCAAGATCG ATGATCCCAC AGACTCCAAG CCTGAGGACT GGGACAAGCC CGAGCATATC
4741  CCTGACCCTG ATGCTAAGAA GCCCGAGGAC TGGGATGAAG AGATGGACGG AGAGTGGGAA
4801  CCCCCAGTGA TTCAGAACCC TGAGTACAAG GGTGAGTGGA AGCCCCGGCA GATCGACAAC
4861  CCAGATTACA AGGGCACTTG GATCCACCCA GAAATTGACA ACCCCGAGTA TTCTCCCGAT
4921  CCCAGTATCT ATGCCTATGA TAACTTTGGC GTGCTGGGCC TGGACCTCTG GCAGGTCAAG
4981  TCTGGCACCA TCTTTGACAA CTTCCTCATC ACCAACGATG AGGCATACGC TGAGGAGTTT
5041  GGCAACGAGA CGTGGGGCGT AACAAAGGCA GCAGAGAAAC AAATGAAGGA CAAACAGGAC
5101  GAGGAGCAGA GGCTTAAGGA GGAGGAAGAA GACAAGAAAC GCAAAGAGGA GGAGGAGGCA
5161  GAGGACAAGG AGGATGATGA GGACAAAGAT GAGGATGAGG AGGATGAGGA GGACAAGGAG
5221  GAAGATGAGG AGGAAGATGT CCCCGGCCAG GCCAAGGACG AGCTGgaatt CATGCATGGA
5281  GATACACCTA CATTGCATGA ATATATGTTA GATTTGCAAC CAGAGACAAC TGATCTCTAC
5341  GGTTATGGGC AA TTAAA TGA CAGCTCAGAG GAGGAGGA TG AAA TAGA TGG TCCAGCTGGA
5401  CAAGCAGAAC CGGACAGAGC CCA TTACAAT A TTGTAACCT TTTGTTGCAA GTGTGACTCT
5461  ACGCTTCGGT TGTGCGTACA AAGCACACAC GTAGACA TTC GTACTTTGGA AGACCTGTTA
5521  ATGGGCACAC TAGGAATTGT GTGCCCCATC TGTTCTCAGA A4CC4TAAαα at CCagat Ct
5581  ttttccctct gccaaaaatt atggggacat catgaagccc cttgagcatc tgacttctgg
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5641  ctaataaagg aaatttattt tcattgcaat agtgtgttgg aatttttttgt gtctctcact
5701  cggaaggaca tatgggaggg caaatcattt aaaacatcag aatgagtatt tggtttagag
5761  tttggcaaca tatgcccatt cttccgcttc ctcgctcact gactcgctgc gctcggtcgt
5821  tcggctgcgg cgagcggtat cagctcactc aaaggcggta atacggttat ccacagaatc
5881  aggggataac gcaggaaaga acatgtgagc aaaaggccag caaaaggcca ggaaccgtaa
5941  aaaggccgcg ttgctggcgt ttttccatag      5970
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Table 2 below describes the structure of the above plasmid (see also Figure 12 for diagram).

TABLE 2

Plasmid Position	Genetic Construct	Source of Construct
5970-0823	E. coli ORI (ColEI)	pBR / *E. coli* -derived
0837-0881	portion of transposase (tpnA)	Common plasmid sequence Tn5/Tn903
0882-1332	β-Lactamase (Amp^R)	pBRpUC derived plasmid
1331-2496	AphA (Kan^R)	Tn903
2509-2691	P3 Promoter DNA binding site	Tn3/pBR322
2692-2926	pUC backbone	Common plasmid sequence pBR322-derived
2931-4009	NF1 binding and promoter	HHV-5(HCMV UL-10 IE1 gene)
4010-4014	Poly-cloning site	Common plasmid sequence
4015 - 5265	**Calreticulin (CRT)**	**Human Calreticulin**
5266-5271	GAATTC plasmid sequence	Remain after cloning
5272-5568	**dE7 gene (detoxified partial)**	**HPV-16 (E7 gene)** incl. stop codon
5569-5580	Poly-cloning site	Common plasmid sequence
551-5970	Poly-Adenylation site	Mammalian signal, pHCMV-derived

Homologues or variants of CRT's as described herein, may also be used, provided that they have the requisite biological activity. These include various substitutions, deletions, or additions of the amino acid or nucleic acid sequences. Due to code degeneracy, for example, there may be considerable variation in nucleotide sequences encoding the same amino acid sequence.

A functional derivative of CRT retains measurable CRT-like activity, preferably that of promoting immunogenicity of one or more antigenic epitopes fused thereto by promoting presentation by class I pathways. "Functional derivatives" encompass "variants" and "fragments" regardless of whether the terms are used in the conjunctive or the alternative herein.

The term "chimeric" or "fusion" polypeptide or protein refers to a composition comprising at least one polypeptide or peptide sequence or domain that is chemically bound in a linear fashion with a second polypeptide or peptide domain. One embodiment of this invention is an isolated or recombinant nucleic acid molecule encoding a fusion protein comprising at least two domains, wherein the first domain comprises an IPP and the second domain comprises an antigenic epitope, *e.g.,* an MHC class I-

binding peptide epitope. The "fusion" can be an association generated by a peptide bond, a chemical linking, a charge interaction *(e.g.,* electrostatic attractions, such as salt bridges, H-bonding, *etc.)* or the like. If the polypeptides are recombinant, the "fusion protein" can be translated from a common mRNA. Alternatively, the compositions of the domains can be linked by any chemical or electrostatic means. The chimeric molecules of the invention *(e.g.,* targeting polypeptide fusion proteins) can also include additional sequences, *e.g.,* linkers, epitope tags, enzyme cleavage recognition sequences, signal sequences, secretion signals, and the like. Alternatively, a peptide can be linked to a carrier simply to facilitate manipulation or identification/ location of the peptide.

Also included is a "functional derivative" of an IPP, preferably CRT (or of its coding sequence) which refers to an amino acid substitution variant, a "fragment," *etc.,* of the protein, which terms are defined below. A functional derivative of an IPP retains measurable activity, preferably that is manifest as promoting immunogenicity of one or more antigenic epitopes fused thereto or co-administered therewith. "Functional derivatives" encompass "variants" and "fragments" regardless of whether the terms are used in the conjunctive or the alternative herein.

A functional homologue must possess the above biochemical and biological activity. In view of this functional characterization, use of homologous proteins including proteins not yet discovered, fall within the scope of the invention if these proteins have sequence similarity and the recited biochemical and biological activity.

To determine the percent identity of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes *(e.g.,* gaps can be introduced in one or both of a first and a second amino acid or nucleic acid sequence for optimal alignment and non-homologous sequences can be disregarded for comparison purposes). In a preferred method of alignment, Cys residues are aligned.

In a preferred embodiment, the length of a sequence being compared is at least 30%, preferably at least 40%, more preferably at least 50%, even more preferably at least 60%, and even more preferably at least 70%, 80%, or 90% of the length of the reference sequence *(e.g.,* CRT, SEQ ID NO: 10). The amino acid residues (or nucleotides) at corresponding amino acid (or nucleotide) positions are then compared. When a position in the first sequence is occupied by the same amino acid residue (or nucleotide) as the corresponding position in the second sequence, then the molecules are identical at that position (as used herein amino acid or nucleic acid "identity" is equivalent to amino acid or nucleic acid "homology"). The percent identity between the two sequences is a function of the number of identical positions shared by the sequences, taking into account the number of gaps, and the length of each gap, which need to be introduced for optimal alignment of the two sequences.

The comparison of sequences and determination of percent identity between two sequences can be accomplished using a mathematical algorithm. In a preferred embodiment, the percent identity between two amino acid sequences is determined using the Needleman and Wunsch *(J. MoI. Biol.* 45:444-453 (1970) algorithm which has been incorporated into the GAP program in the GCG software package (available at http://www.gcg.com), using either a Blossom 62 matrix or a PAM250 matrix, and a gap weight of 16, 14, 12, 10, 8, 6, or 4 and a length weight of 1, 2, 3, 4, 5, or 6. In yet another preferred embodiment, the percent identity between two nucleotide sequences is determined using the GAP program in the GCG software package (available at http://www.gcg.com), using a NWSgapdna.CMP matrix and a gap weight of 40, 50, 60, 70, or 80 and a length weight of 1, 2, 3, 4, 5, or 6. In another embodiment, the percent identity between two amino acid or nucleotide sequences is determined using the algorithm of E. Meyers and W. Miller (CABIOS, 4:1 1-17 (1989)) which has been incorporated into the ALIGN program (version 2.0), using a PAM120 weight residue table, a gap length penalty of 12 and a gap penalty of 4.

The nucleic acid and protein sequences of the present invention can further be used as a "query sequence" to perform a search against public databases, for example, to identify other family members or related sequences. Such searches can be performed using the NBLAST and XBLAST programs (version 2.0) of Altschul *et al.* (1990) *J. MoI. Biol.* 275:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to **CRT** nucleic acid molecules. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to HVP22 protein molecules. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs *(e.g.,* XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov.

Thus, a homologue of CRT described above is characterized as having (a) functional activity of native CRT and (b) sequence similarity to a native CRT protein (such as SEQ ID NO:9) when determined as above, of at least about 20% (at the amino acid level), preferably at least about 40%, more preferably at least about 70%, even more preferably at least about 90%.

It is within the skill in the art to obtain and express such a protein using DNA probes based on the disclosed sequences of CRT. Then, the fusion protein's biochemical and biological activity can be tested readily using art-recognized methods such as those described herein, for example, a T cell proliferation, cytokine secretion or a cytolytic assay, or an *in vivo* assay of tumor protection or tumor therapy. A biological assay of the stimulation of antigen-specific T cell reactivity will indicate whether the homologue has the requisite activity to qualify as a "functional" homologue.

A "variant" refers to a molecule substantially identical to either the full protein or to a fragment thereof in which one or more amino acid residues have been replaced (substitution variant) or which has one or several residues deleted (deletion variant) or added (addition variant). A "fragment" of CRT refers to any subset of the molecule, that is, a shorter polypeptide of the full-length protein.

A number of processes can be used to generate fragments, mutants and variants of the isolated DNA sequence. Small subregions or fragments of the nucleic acid encoding the spreading protein, for example 1-30 bases in length, can be prepared by standard, chemical synthesis. Antisense oligonucleotides and primers for use in the generation of larger synthetic fragment.

A preferred group of variants are those in which at least one amino acid residue and preferably, only one, has been substituted by different residue. For a detailed description of protein chemistry and structure, see Schulz, GE *et ah, Principles of Protein Structure,* Springer-Verlag, New York, 1978, and Creighton, T.E., *Proteins: Structure and Molecular Properties,* W.H. Freeman & Co., San Francisco, 1983, which are hereby incorporated by reference. The types of substitutions that may be made in the protein molecule may be based on analysis of the frequencies of amino acid changes between a homologous protein of different species, such as those presented in Table 1-2 of Schulz *et al. (supra)* and Figure 3-9 of Creighton *(supra).* Based on such an analysis, conservative substitutions are defined herein as exchanges within one of the following five groups:

1.	Small aliphatic, nonpolar or slightly polar residues	Ala, Ser, Thr (Pro, GIy);
2.	Polar, negatively charged residues and their amides	Asp, Asn, GIu, GIn;
3.	Polar, positively charged residues	His, Arg, Lys;
4.	Large aliphatic, nonpolar residues	Met, Leu, He, VaI (Cys)
5.	Large aromatic residues	Phe, Tyr, Trp.

The three amino acid residues in parentheses above have special roles in protein architecture. GIy is the only residue lacking a side chain and thus imparts flexibility to the chain. Pro, because of its unusual geometry, tightly constrains the chain. Cys can participate in disulfide bond formation, which is important in protein folding.

More substantial changes in biochemical, functional (or immunological) properties are made by selecting substitutions that are less conservative, such as between, rather than within, the above five groups. Such changes will differ more significantly in their effect on maintaining (a) the structure of the peptide backbone in the area of the substitution, for example, as a sheet or helical conformation, (b) the charge or hydrophobicity of the molecule at the target site, or (c) the bulk of the side chain. Examples of such substitutions are (i) substitution of GIy and/or Pro by another amino acid or deletion or insertion of GIy or Pro; (ii) substitution of a hydrophilic residue, *e.g.,* Ser or Thr, for (or by) a hydrophobic residue, *e.g.,,* Leu, He, Phe, VaI or Ala; (iii) substitution of a Cys residue for (or by) any other residue;

(iv) substitution of a residue having an electropositive side chain, *e.g.*,, Lys, Arg or His, for (or by) a residue having an electronegative charge, *e.g.*,, GIu or Asp; or (v) substitution of a residue having a bulky side chain, *e.g.*, Phe, for (or by) a residue not having such a side chain, *e.g.*, GIy.

Most acceptable deletions, insertions and substitutions according to the present invention are those that do not produce radical changes in the characteristics of the wild-type or native protein in terms of its intercellular spreading activity and its ability to stimulate antigen specific T cell reactivity to an antigenic epitope or epitopes that are fused to the spreading protein. However, when it is difficult to predict the exact effect of the substitution, deletion or insertion in advance of doing so, one skilled in the art will appreciate that the effect can be evaluated by routine screening assays such as those described here, without requiring undue experimentation.

Whereas shorter chain variants can be made by chemical synthesis, for the present invention, the preferred longer chain variants are typically made by site-specific mutagenesis of the nucleic acid encoding the polypeptide, expression of the variant nucleic acid in cell culture, and, optionally, purification of the polypeptide from the cell culture, for example, by immunoaffinity chromatography using specific antibody immobilized to a column (to absorb the variant by binding to at least one epitope).

In the above composition the CRT or other stress protein preferably acts in potentiating an immune response by promoting: processing of the linked antigenic polypeptide via the MHC class I pathway or targeting of a cellular compartment that increases the processing. This basic strategy may be combined with an additional strategy pioneered by the present inventors and colleagues, that involve linking DNA encoding another protein, generically termed a "targeting polypeptide, to the antigen-encoding DNA. Again, for the sake of simplicity, the DNA encoding such a targeting polypeptide will be referred to herein as a "targeting DNA." That strategy has been shown to be effective in enhancing the potency of the vectors carrying only antigen-encoding DNA. See for example, the following PCT publications by Wu *et al*: WO 01/29233; WO 02/009645; WO 02/061 113; WO 02/074920; and WO 02/12281, all of which are incorporated by reference in their entirety. The other strategies include the use of DNA encoding polypeptides that promote or enhance:

(a) development, accumulation or activity of antigen presenting cells or targeting of antigen to compartments of the antigen presenting cells leading to enhanced antigen presentation;

(b) intercellular transport and spreading of the antigen; or

(c) any combination of (a) and (b).

(d) sorting of the lysosome-associated membrane protein type 1 (Sig/LAMP-1).

The strategy includes use of:

(e) a viral intercellular spreading protein selected from the group of herpes simplex virus-1 VP22 protein, Marek's disease virus UL49 (see WO 02/09645), protein or a functional homologue or derivative thereof;

(f) other endoplasmic reticulum chaperone polypeptides selected from the group of CRT-like molecules ER60, GRP94, gp96, or a functional homologue or derivative thereof (see WO 02/12281, hereby incorporated by reference;

(g) a cytoplasmic translocation polypeptide domains of a pathogen toxin selected from the group of domain II of *Pseudomonas* exotoxin ETA or a functional homologue or derivative thereof;

(h) a polypeptide that targets the centrosome compartment of a cell selected from γ-tubulin or a functional homologue or derivative thereof; or

(i) a polypeptide that stimulates dendritic cell precursors or activates dendritic cell activity selected from the group of GM-CSF, Flt3-ligand extracellular domain, or a functional homologue or derivative thereof; or .

(j) a costimulatory signal, such as a B7 family protein, including B7-DC (see U.S. Serial No. 09/794,210), B7.1, B7.2, soluble CD40, *etc.).*

(k) an anti-apoptotic polypeptide preferably selected from the group consisting of (1) BCL-xL, (2) BCL2, (3) XIAP, (4) FLICEc-s, (5) dominant-negative caspase-8, (6) dominant negative caspase-9, (7) SPI-6, and (8) a functional homologue or derivative of any of (l)-(7). (See WO 2005/047501).

The details of the various targeting polypeptide strategies will not be discussed in detail herein. Of the IPP's above, the preferred alternatives to CRT are the other ER chaperone polypeptide exemplified by ER60, GRP94 or gp96, well-characterized ER chaperone polypeptide that representatives of the HSP90 family of stress-induced proteins (see WO 02/012281)

The experiments described herein demonstrate that the methods of the invention can enhance a cellular immune response, particularly, tumor-destructive CTL reactivity, induced by a DNA vaccine encoding an epitope of a human pathogen. Human HPV-16 E7 was used as a model antigen for vaccine development because human papillomaviruses (HPVs), particularly HPV- 16, are associated with most human cervical cancers. The oncogenic HPV proteins E7 and E6 are important in the induction and maintenance of cellular transformation and co-expressed in most HPV-containing cervical cancers and their precursor lesions. Therefore, cancer vaccines, such as the compositions of the invention, that target E7 or E6 can be used to control of HPV-associated neoplasms (Wu, T-C, *Curr Opin Immunol. 6:146-54,* 1994).

However, as noted, the present invention is not limited to the exemplified antigen(s). Rather, one of skill in the art will appreciate that the same results are expected for any antigen (and epitopes

thereof) for which a T cell-mediated response is desired. The response so generated will be effective in providing protective or therapeutic immunity, or both, directed to an organism or disease in which the epitope or antigenic determinant is involved —for example as a cell surface antigen of a pathogenic cell or an envelope or other antigen of a pathogenic virus, or a bacterial antigen, or an antigen expressed as or as part of a pathogenic molecule.

Thus, in one embodiment, the antigen *(e.g.,* the MHC class I-binding peptide epitope) is derived from a pathogen, *e.g.,* it comprises a peptide expressed by a pathogen. The pathogen can be a virus, such as, *e.g.,* a papilloma virus, a herpesvirus, a retrovirus *(e.g.,* an immunodeficiency virus, such as HIV-I), an adenovirus, and the like. The papilloma virus can be a human papilloma virus; for example, the antigen *(e.g.,* the Class I-binding peptide) can be derived from an HPV-16 E6 or E7 polypeptide. In a preferred embodiment, the HPV-16 E6 or E7 polypeptide used as an immunogen is substantially non-oncogenic, *i.e.,* it does not bind retinoblastoma polypeptide (pRB) or binds pRB with such low affinity that the HPV-16 E7 polypeptide is effectively non-oncogenic when expressed or delivered *in vivo,* which is accomplished as described herein.

In alternative embodiments, the pathogen is a bacteria, such as *Bordetella pertussis; Ehrlichia chaffeensis; Staphylococcus aureus; Toxoplasma gondii; Legionella pneumophila; Brucella suis; Salmonella enterica; Mycobacterium avium; Mycobacterium tuberculosis; Listeria monocytogenes; Chlamydia trachomatis; Chlamydia pneumoniae; Rickettsia rickettsii;* or, a fungus, such as, *e.g., Paracoccidioides brasiliensis;* or other pathogen, *e.g., Plasmodium falciparum.*

In another embodiment, the MHC class I-binding antigenic peptide epitope is derived from a tumor cell. The tumor cell-derived peptide epitope can comprise a tumor associated antigen, *e.g.,* a tumor specific antigen, such as, *e.g.,* a HER-2/neu antigen, or one of a number of known melanoma antigens, *etc.*.

In one embodiment, the isolated or recombinant nucleic acid molecule is operatively linked to a promoter, such as, *e.g.,* a constitutive, an inducible or a tissue-specific promoter. The promoter can be expressed in any cell, including cells of the immune system, including, *e.g.,* antigen presenting cells (APCs), *e.g.,* in a constitutive, an inducible or a tissue-specific manner.

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art of this invention. As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

The term "antigen" or "immunogen" as used herein refers to a compound or composition comprising a peptide, polypeptide or protein which is "antigenic" or "immunogenic" when administered (or expressed *in vivo* by an administered nucleic acid, *e.g.,* a DNA vaccine) in an appropriate amount (an "immunogenically effective amount"), *i.e.,* capable of inducing, eliciting, augmenting or boosting a

cellular and/or humoral immune response either alone or in combination or linked or fused to another substance (which can be administered at once or over several intervals). An immunogenic composition can comprise an antigenic peptide of at least about 5 amino acids, a peptide of 10 amino acids in length, a polypeptide fragment of 15 amino acids in length, 20 amino acids in length or longer. Smaller immunogens may require presence of a "carrier" polypeptide *e.g.,* as a fusion protein, aggregate, conjugate or mixture, preferably linked (chemically or otherwise) to the immunogen. The immunogen can be recombinantly expressed from a vaccine vector, which can be naked DNA comprising the immunogen's coding sequence operably linked to a promoter, *e.g.,* an expression cassette as described herein. The immunogen includes one or more antigenic determinants or epitopes which may vary in size from about 3 to about 15 amino acids.

The term "vaccine" is used interchangeably with "immunogen" when referring to the DNA compositions of the present invention. Similarly, the terms "vaccinate" and "immunize" are used interchangeably here.

The term "epitope" as used herein refers to an antigenic determinant or antigenic site that interacts with an antibody or a T cell receptor (TCR), *e.g.,* the MHC class I-binding peptide compositions (or expressed products of the nucleic acid compositions of the invention) used in the methods of the invention. An "antigen" is a molecule or chemical structure that either induces an immune response or is specifically recognized or bound by the product or mediator of an immune response, such as an antibody or a CTL. The specific conformational or stereochemical "domain" to which an antibody or a TCR bind is an "antigenic determinant" or "epitope." TCRs bind to peptide epitopes which are physically associated with a third molecule, a major histocompatibility complex (MHC) class I or class II protein.

The term "recombinant" refers to (1) a nucleic acid or polynucleotide synthesized or otherwise manipulated *in vitro,* (2) methods of using recombinant DNA technology to produce gene products in cells or other biological systems, or (3) a polypeptide encoded by a recombinant nucleic acid. For example, the CRT -encoding nucleic acid or polypeptide, the nucleic acid encoding an MHC class I-binding peptide epitope (antigen) or the peptide itself is preferably be recombinant. "Recombinant means" includes ligation of nucleic acids having various coding regions or domains or promoter sequences from different sources into a single unit in the form of an expression cassette or vector for expression of the coding sequences in the vectors resulting in production of the encoded polypeptide.

GENERAL RECOMBINANT DNA METHODS

Basic texts disclosing general methods of molecular biology, all of which are incorporated by reference, include: Sambrook, J *et al, Molecular Cloning: A Laboratory Manual,* 2nd Edition, Cold Spring Harbor Press, Cold Spring Harbor, NY, 1989; Ausubel, FM *et al. Current Protocols in Molecular*

Biology, Vol. 2, Wiley-Interscience, New York, (current edition); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); Glover, DM, ed, *DNA Cloning: A Practical Approach,* vol. I & II, IRL Press, 1985; Albers, B. *et al, Molecular Biology of the Cell,* 2nd Ed., Garland Publishing, Inc., New York, NY (1989); Watson, JD *et al, Recombinant DNA,* 2nd Ed., Scientific American Books, New York, 1992; and Old, RW *et al, Principles of Gene Manipulation: An Introduction to Genetic Engineering,* 2nd Ed., University of California Press, Berkeley, CA (1981).

Techniques for the manipulation of nucleic acids, such as, *e.g.,* generating mutations in sequences, subcloning, labeling probes, sequencing, hybridization and the like are well described in the scientific and patent literature. See, *e.g.,* Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), VOIS. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc., New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Tijssen, ed. Elsevier, N.Y. (1993).

Nucleic acids, vectors, capsids, polypeptides, and the like can be analyzed and quantified by any of a number of general means well known to those of skill in the art. These include, *e.g.,* analytical biochemical methods such as NMR, spectrophotometry, radiography, electrophoresis, capillary electrophoresis, high performance liquid chromatography (HPLC), thin layer chromatography (TLC), and hyperdiffusion chromatography, various immunological methods, *e.g.* fluid or gel precipitin reactions, immunodiffusion, immuno-electrophoresis, radioimmunoassays (RIAs), enzyme-linked immunosorbent assays (ELISAs), immunofluorescence assays, Southern analysis, Northern analysis, dot-blot analysis, gel electrophoresis (e.g., SDS-PAGE), RT-PCR, quantitative PCR, other nucleic acid or target or signal amplification methods, radiolabeling, scintillation counting, and affinity chromatography.

Amplification of Nucleic Acids

Oligonucleotide primers can be used to amplify nucleic acids to generate fusion protein coding sequences used to practice the invention, to monitor levels of vaccine after *in vivo* administration *(e.g.,* levels of a plasmid or virus), to confirm the presence and phenotype of activated CTLs, and the like. The skilled artisan can select and design suitable oligonucleotide amplification primers using known sequences. Amplification methods are also well known in the art, and include, *e.g.,* polymerase chain reaction, PCR *(PCR Protocols, A Guide to Methods and Applications,* ed. Innis, Academic Press, N.Y. (1990) and *PCR Strategies* (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (Wu (1989) *Genomics 4:560;* Landegren (1988) *Science 241:1077;* Barringer (1990) Gene 89:1 17); transcription amplification (Kwoh (1989) *Proc. Natl Acad. Set USA 86:1 173);* and, self-sustained sequence replication (Guatelli (1990) *Proc. Natl. Acad. Sci. USA* §7:1874); Qβ replicase amplification

(Smith (1997) *J. Clin. Microbiol.* 35:1477-1491; Burg (1996) *Mol. Cell. Probes 10:251-211)* and other RNA polymerase mediated techniques (NASBA, Cangene, Mississauga, Ontario; Berger (1987) *Methods Enzymol.* 752:307-316; U.S. Pats No. 4,683,195 and 4,683,202; Sooknanan (1995) *Biotechnology* 73:563-564).

Unless otherwise indicated, a particular nucleic acid sequence is intended to encompasses conservative substitution variants thereof *(e.g.,* degenerate codon substitutions) and a complementary sequence. The term "nucleic acid" is synonymous with "polynucleotide" and is intended to include a gene, a cDNA molecule, an mRNA molecule, as well as a fragment of any of these such as an oligonucleotide, and further, equivalents thereof (explained more fully below). Sizes of nucleic acids are stated either as kilobases (kb) or base pairs (bp). These are estimates derived from agarose or polyacrylamide gel electrophoresis (PAGE), from nucleic acid sequences which are determined by the user or published. Protein size is stated as molecular mass in kilodaltons (kDa) or as length (number of amino acid residues). Protein size is estimated from PAGE, from sequencing, from presumptive amino acid sequences based on the coding nucleic acid sequence or from published amino acid sequences.

Specifically, cDNA molecules encoding the amino acid sequence corresponding to the fusion polypeptide of the present invention or fragments or derivatives thereof can be synthesized by the polymerase chain reaction (PCR) (see, for example, U.S. 4,683,202) using primers derived the sequence of the protein disclosed herein. These cDNA sequences can then be assembled into a eukaryotic or prokaryotic expression vector and the resulting vector can be used to direct the synthesis of the fusion polypeptide or its fragment or derivative by appropriate host cells, for example COS or CHO cells.

This invention includes isolated nucleic acids having a nucleotide sequence encoding the novel fusion polypeptides that comprise a translocation polypeptide and an antigen, fragments thereof or equivalents thereof. The term nucleic acid as used herein is intended to include such fragments or equivalents. The nucleic acid sequences of this invention can be DNA or RNA.

A cDNA nucleotide sequence the fusion polypeptide can be obtained by isolating total mRNA from an appropriate cell line. Double stranded cDNA is prepared from total mRNA. cDNA can be inserted into a suitable plasmid, bacteriophage or viral vector using any one of a number of known techniques.

In reference to a nucleotide sequence, the term "equivalent" is intended to include sequences encoding structurally homologous and/or a functionally equivalent proteins. For example, a natural polymorphism in a nucleotide sequence encoding an anti-apoptotic polypeptide according to the present invention (especially at the third base of a codon) may be manifest as "silent" mutations which do not change the amino acid sequence. Furthermore, there may be one or more naturally occurring isoforms or related, immunologically cross-reactive family members of these proteins. Such isoforms or family

members are defined as proteins that share function amino acid sequence similarity to the reference polypeptide.

Fragment of Nucleic Acid

A fragment of a nucleic acid sequence is defined as a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length CRT polypeptide, antigenic polypeptide, or the fusion thereof. This invention includes such nucleic acid fragments that encode polypeptides which retain (1) the ability of the fusion polypeptide to induce increases in frequency or reactivity of T cells, preferably CD8+ T cells, that are specific for the antigen part of the fusion polypeptide.

For example, a nucleic acid fragment as intended herein encodes an antigen, or encodes CRT or a homologue, domain, or fragment thereof, that retains the ability to improve the immunogenicity of an antigen-only DNA vaccine when administered as a chimeric DNA with antigen-encoding sequence.

Generally, the nucleic acid sequence encoding a fragment of an anti-apoptotic polypeptide comprises of nucleotides from the sequence encoding the mature protein (or an active fragment thereof).

Nucleic acid sequences of this invention may also include linker sequences, natural or modified restriction endonuclease sites and other sequences that are useful for manipulations related to cloning, expression or purification of encoded protein or fragments. These and other modifications of nucleic acid sequences are described herein or are well-known in the art.

The techniques for assembling and expressing DNA coding sequences for translocation types of proteins, and DNA coding sequences for antigenic polypeptides, include synthesis of oligonucleotides, PCR, transforming cells, constructing vectors, expression systems, and the like; these are well-established in the art such that those of ordinary skill are familiar with standard resource materials, specific conditions and procedures.

EXPRESSION VECTORS AND HOST CELLS

This invention includes an expression vector comprising a nucleic acid sequence encoding (a) an antigen linked to (b) an IPP and operably linked to at least one regulatory sequence, which includes a promoter that is expressable in a eukaryotic cell, preferably in a mammalian cells, more preferably in a human cell.

The term "expression vector" or "expression cassette" as used herein refers to a nucleotide sequence which is capable of affecting expression of a protein coding sequence in a host compatible with such sequences. Expression cassettes include at least a promoter operably linked with the polypeptide coding sequence; and, optionally, with other sequences, *e.g.*, transcription termination signals. Additional factors necessary or helpful in effecting expression may also be included, *e.g.*, enhancers.

"Operably linked" means that the coding sequence is linked to a regulatory sequence in a manner that allows expression of the coding sequence. Known regulatory sequences are selected to direct expression of the desired protein in an appropriate host cell. Accordingly, the term "regulatory sequence" includes promoters, enhancers and other expression control elements. Such regulatory sequences are described in, for example, Goeddel, *Gene Expression Technology. Methods in Enzymology,* vol. 185, Academic Press, San Diego, Calif. (1990)).

Thus, expression cassettes include plasmids, recombinant viruses, any form of a recombinant "naked DNA" vector, and the like. A "vector" comprises a nucleic acid which can infect, transfect, transiently or permanently transduce a cell. It will be recognized that a vector can be a naked nucleic acid, or a nucleic acid complexed with protein or lipid. The vector optionally comprises viral or bacterial nucleic acids and/or proteins, and/or membranes *{e.g.,* a cell membrane, a viral lipid envelope, *etc.).* Vectors include replicons (e.g., RNA replicons), bacteriophages) to which fragments of DNA may be attached and become replicated. Vectors thus include, but are not limited to RNA, autonomous self-replicating circular or linear DNA or RNA, *e.g.,* plasmids, viruses, and the like (U.S. Patent No. 5,217,879), and includes both the expression and nonexpression plasmids. Where a recombinant cell or culture is described as hosting an "expression vector" this includes both extrachromosomal circular and linear DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

Those skilled in the art appreciate that the particular design of an expression vector of this invention depends on considerations such as the host cell to be transfected and/or the type of protein to be expressed.

The present expression vectors comprise the full range of nucleic acid molecules encoding the various embodiments of the fusion polypeptide and its functional derivatives (defined herein) including polypeptide fragments, variants, *etc.*

Such expression vectors may be used to transfect host cells *(in vitro, ex vivo* or *in vivo)* for expression of the DNA and production of the encoded proteins which include fusion proteins or peptides. It will be understood that a genetically modified cell expressing the fusion polypeptide may transiently express the exogenous DNA for a time sufficient for the cell to be useful for its stated purpose.

The present in invention provides methods for producing the fusion polypeptides, fragments and derivatives. For example, a host cell transfected with a nucleic acid vector that encodes the fusion polypeptide is cultured under appropriate conditions to allow expression of the polypeptide.

Host cells may also be transfected with one or more expression vectors that singly or in combination comprise (a) DNA encoding at least a portion of the fusion polypeptide and (b) DNA encoding at least a portion of a second protein, preferably an antigen, so that the host cells produce yet further fusion polypeptides.

A culture typically includes host cells, appropriate growth media and other byproducts. Suitable culture media are well known in the art. The fusion polypeptide can be isolated from medium or cell lysates using conventional techniques for purifying proteins and peptides, including ammonium sulfate precipitation, fractionation column chromatography (e.g. ion exchange, gel filtration, affinity chromatography, *etc.)* and/or electrophoresis (see generally, "Enzyme Purification and Related Techniques", *Meth Enzymol,* 22:233-577 (1971)). Once purified, partially or to homogeneity, the recombinant polypeptides of the invention can be utilized in pharmaceutical compositions as described in more detail herein.

The term "isolated" as used herein, when referring to a molecule or composition, such as a translocation polypeptide or a nucleic acid coding therefor, means that the molecule or composition is separated from at least one other compound (protein, other nucleic acid, *etc.)* or from other contaminants with which it is natively associated or becomes associated during processing. An isolated composition can also be substantially pure. An isolated composition can be in a homogeneous state and can be dry or in aqueous solution. Purity and homogeneity can be determined, for example, using analytical chemical techniques such as polyacrylamide gel electrophoresis (PAGE) or high performance liquid chromatography (HPLC). Even where a protein has been isolated so as to appear as a homogenous or dominant band in a gel pattern, there are trace contaminants which co-purify with it.

Host cells transformed or transfected to express the fusion polypeptide or a homologue or functional derivative thereof are within the scope of the invention. For example, the fusion polypeptide may be expressed in yeast, or mammalian cells such as Chinese hamster ovary cells (CHO) or, preferably human cells. Preferred cells for expression according to the present invention are APCs most preferably, DCs. Other suitable host cells are known to those skilled in the art.

Expression in eukaryotic cells leads to partial or complete glycosylation and/or formation of relevant inter- or intra-chain disulfide bonds of the recombinant protein.

Often, in fusion expression vectors, a nucleotide sequence encoding a proteolytic cleavage site is introduced at the junction of the reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein. Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Amrad Corp., Melbourne, Australia), pMAL (New England Biolabs) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase, maltose E

binding protein, or protein A, respectively, to the target recombinant protein. Inducible non-fusion expression vectors include pTrc (Amann *et al, Gene* 69:301-15, 1988) and pET 1Id (Studier *et al, Gene Expression Technology: Meth Enzymol 185:60-89,* Academic Press, 1990).

Vector Construction

Construction of suitable vectors comprising the desired coding and control sequences employs standard ligation and restriction techniques which are well understood in the art. Isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and re-ligated in the form desired. The sequences of several preferred plasmid vectors, with and without inserted coding sequences, have been disclosed above.

The DNA sequences which form the vectors are available from a number of sources. Backbone vectors and control systems are generally found on available "host" vectors which are used for the bulk of the sequences in construction. For the pertinent coding sequence, initial construction may be, and usually is, a matter of retrieving the appropriate sequences from cDNA or genomic DNA libraries. However, once the sequence is disclosed it is possible to synthesize the entire gene sequence *in vitro* starting from the individual nucleotide derivatives. The entire gene sequence for genes of sizeable length, *e.g.,* 500-1000 bp may be prepared by synthesizing individual overlapping complementary oligonucleotides and filling in single stranded nonoverlapping portions using DNA polymerase in the presence of the deoxyribonucleotide triphosphates. This approach has been used successfully in the construction of several genes of known sequence.

Synthetic oligonucleotides are prepared by either the phosphotriester method as described by references cited above or the phosphoramidite method (Beaucage, SL *et al, TetLett* 22:1859, 1981; Matteucci, MD *et al,, J Am Chem Soc* /03:3185, 1981) and can be prepared using commercially available automated oligonucleotide synthesizers. Kinase treatment of single strands prior to annealing or for labeling is by conventional methods.

Once the components of the desired vectors are thus available, they can be excised and ligated using standard restriction and ligation procedures. Site-specific DNA cleavage is performed by treating with the suitable restriction enzyme (or enzymes) under conditions which are understood in the art, and the particulars of which are specified by the manufacturer of these commercially available restriction enzymes. See, *e.g.,* New England Biolabs, Product Catalog; *Meth Enzymol.* (55:499-560, 1980.

Restriction cleaved fragments may be blunt ended by treating with the large fragment of *E. coli* DNA polymerase I (Klenow) in the presence of the four deoxynucleotide triphosphates (dNTPs) using conventional methods and conditions. Ligations are performed using conventional methods. In vector construction employing "vector fragments", the fragment is commonly treated with bacterial or mammalian alkaline phosphatase to remove the 5' phosphate and prevent self-ligation. Alternatively, re-

ligation can be prevented in vectors which have been double digested by additional restriction enzyme and separation of the unwanted fragments.

Any of a number of methods are used to introduce mutations into the coding sequence to generate the variants of the invention. These mutations include simple deletions or insertions, systematic deletions, insertions or substitutions of clusters of bases or substitutions of single bases.

For example, modifications of DNA sequences are created by site-directed mutagenesis, a well-known technique for which protocols and reagents are commercially available (Zoller, MJ *et al, Nucleic Acids Res* 10:6487-500, 1982; Adelman, JP *et al., DNA* 2:183-193, 1983). Using conventional methods, transformants are selected based on the presence of a selectable marker such as an antibiotic resistance gene depending on the mode of plasmid construction.

Vector DNA can be introduced into mammalian cells via conventional techniques such as calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming host cells can be found in Sambrook *et al. supra* and other standard texts. In fusion expression vectors, a proteolytic cleavage site may be introduced at the junction of two sequences (such as a reporter group and the target protein to enable separation of the target protein from the reporter group subsequent to purification of the fusion protein). Proteolytic enzymes for such cleavage and their recognition sequences include Factor Xa, thrombin and enterokinase.

Promoters and Enhancers

A promoter region of a DNA or RNA molecule binds RNA polymerase and promotes the transcription of an "operably linked" nucleic acid sequence. As used herein, a "promoter sequence" is the nucleotide sequence of the promoter which is found on that strand of the DNA or RNA which is transcribed by the RNA polymerase. Two sequences of a nucleic acid molecule, such as a promoter and a coding sequence, are "operably linked" when they are linked to each other in a manner which permits both sequences to be transcribed onto the same RNA transcript or permits an RNA transcript begun in one sequence to be extended into the second sequence. Thus, two sequences, such as a promoter sequence and a coding sequence of DNA or RNA are operably linked if transcription commencing in the promoter sequence will produce an RNA transcript of the operably linked coding sequence. In order to be "operably linked" it is not necessary that two sequences be immediately adjacent to one another in the linear sequence.

The preferred promoter sequences of the present invention must be operable in mammalian cells and may be either eukaryotic or viral promoters. Although preferred promoters are described in the Examples, other useful promoters and regulatory elements are discussed below. Suitable promoters may be inducible, repressible or constitutive. A "constitutive" promoter is one which is active under most

conditions encountered in the cell's environmental and throughout development. An "inducible" promoter is one which is under environmental or developmental regulation. A "tissue specific" promoter is active in certain tissue types of an organism. An example of a constitutive promoter is the viral promoter MSV-LTR, which is efficient and active in a variety of cell types, and, in contrast to most other promoters, has the same enhancing activity in arrested and growing cells. Other preferred viral promoters include that present in the CMV-LTR (from cytomegalovirus) (Bashart, M. *et al, Cell 41:521,* 1985) or in the RSV-LTR (from Rous sarcoma virus) (Gorman, CM., *Proc. Natl. Acad. ScL USA 79:6111,* 1982). Also useful are the promoter of the mouse metallothionein I gene (Hamer, D, *et al, J. MoI. Appl. Gen. /*:273-88, 1982; the TK promoter of Herpes virus (McKnight, S, *Cell 31:355-65,* 1982); the SV40 early promoter (Benoist, C , *et al., Nature* 290:304-10, 1981); and the yeast *gal4* gene promoter (Johnston, SA *et al, Proc. Natl Acad. ScI USA 79:6911-5,* 1982); Silver, PA, *et al, Proc. Natl. Acad. ScL (USA) 81:5951-5,* 1984)). Other illustrative descriptions of transcriptional factor association with promoter regions and the separate activation and DNA binding of transcription factors include: Keegan *et al, Nature 231:699,* 1986; Fields *et al, Nature 340:245,* 1989; Jones, *Cell 61:9,* 1990; Lewin, Cell *61:1161,* 1990; Ptashne *et al, Nature 346:329,* 1990; Adams *et al, Cell 72:306,* 1993.

The promoter region may further include an octamer region which may also function as a tissue specific enhancer, by interacting with certain proteins found in the specific tissue. The enhancer domain of the DNA construct of the present invention is one which is specific for the target cells to be transfected, or is highly activated by cellular factors of such target cells. Examples of vectors (plasmid or retrovirus) are disclosed in (Roy-Burman *et al,* U.S. Patent No. 5,1 12,767). For a general discussion of enhancers and their actions in transcription, see, Lewin, BM, *Genes IV,* Oxford University Press pp. 552-576, 1990 (or later edition). Particularly useful are retroviral enhancers (e.g., viral LTR) that is preferably placed upstream from the promoter with which it interacts to stimulate gene expression. For use with retroviral vectors, the endogenous viral LTR may be rendered enhancer-less and substituted with other desired enhancer sequences which confer tissue specificity or other desirable properties such as transcriptional efficiency.

Nucleic acids of the invention can also be chemically synthesized using standard techniques, including solid-phase synthesis which, like peptide synthesis, has been fully automated with commercially available DNA synthesizers (Itakura U.S. Pats. No. 4,598,049, 4,401,796 and 4,373,071; Caruthers *et al* U.S. Pat. No. 4,458,066.

THERAPEUTIC COMPOSITIONS AND THEIR ADMINISTRATION

A vaccine composition comprising the nucleic acid encoding the antigen in a fusion polypeptide with an IPP, here CRT or a homologue or derivative thereof, a particle comprising the nucleic acid or a cell expressing this nucleic acid, is administered to a mammalian subject. The vaccine composition is

administered in a pharmaceutically acceptable carrier in a biologically-effective or a therapeutically-effective amount.

Certain preferred conditions are disclosed in the Examples. The composition may be given alone or in combination with another protein or peptide such as an immunostimulatory molecule. Treatment may include administration of an adjuvant, used in its broadest sense to include any nonspecific immune stimulating compound such as an interferon. Adjuvants contemplated herein include resorcinols, non-ionic surfactants such as polyoxyethylene oleyl ether and n-hexadecyl polyethylene ether.

A therapeutically effective amount is a dosage that, when given for an effective period of time, achieves the desired immunological or clinical effect.

A therapeutically active amount of a nucleic acid encoding the fusion polypeptide may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the peptide to elicit a desired response in the individual. Dosage regimes may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies of the therapeutic situation. A therapeutically effective amounts of the protein, in cell associated form may be stated in terms of the protein or cell equivalents.

Thus an effective amount of the vaccine is between about 1 nanogram and about 1 gram per kilogram of body weight of the recipient, more preferably between about 0.1 μg/kg and about lOmg/kg, more preferably between about 1 μg/kg and about 1 mg/kg. Dosage forms suitable for internal administration preferably contain (for the latter dose range) from about 0.1 μg to 100 μg of active ingredient per unit. The active ingredient may vary from 0.5 to 95% by weight based on the total weight of the composition. Alternatively, an effective dose of cells transfected with the DNA vaccine constructs of the present invention is between about 10^4 and 10^8 cells. Those skilled in the art of immunotherapy will be able to adjust these doses without undue experimentation.

The composition may be administered in a convenient manner, *e.g.,* injection by a convenient and effective route.

Preferred routes for the DNA include (a) intradermal "gene gun" delivery wherein DNA-coated gold particles in an effective amount are delivered using a helium-driven gene gun (BioRad, Hercules, CA) with a discharge pressure set at a known level, *e.g.,* of 400 p.s.i.; (b) i.m. injection using a conventional syringe needle; and (c) use of a needle-free biojector such as the Biojector 2000 (Bioject Inc., Portland, OR) which is an injection device consisting of an injector and a disposable syringe. The orifice size controls the depth of penetration. For example, 50μg of DNA may be delivered using the Biojector with no. 2 syringe nozzle.

Depending on the route of administration, the composition may be coated in a material to protect the compound from the action of enzymes, acids and other natural conditions which may inactivate the compound. Thus it may be necessary to coat the composition with, or co-administer the composition with, a material to prevent its inactivation. For example, an enzyme inhibitors of nucleases or proteases (e.g., pancreatic trypsin inhibitor, diisopropylfluorophosphate and trasylol).or in an appropriate carrier such as liposomes (including water-in-oil-in-water emulsions as well as conventional liposomes (Strejan *et al, J. Neuroimmunol* 7:27, 1984).

As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

Preferred pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Pharmaceutical compositions suitable for injection include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. Isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, sodium chloride may be included in the pharmaceutical composition. In all cases, the composition should be sterile and should be fluid. It should be stable under the conditions of manufacture and storage and must include preservatives that prevent contamination with microorganisms such as bacteria and fungi. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants.

Prevention of the action of microorganisms in the pharmaceutical composition can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like.

Compositions are preferably formulated in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form refers to physically discrete units suited as unitary dosages for a mammalian subject; each unit contains a predetermined quantity of active material *{e.g.,* the nucleic acid

vaccine) calculated to produce the desired therapeutic effect, in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active material and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of, and sensitivity of, individual subjects

For lung instillation, aerosolized solutions are used. In a sprayable aerosol preparations, the active protein may be in combination with a solid or liquid inert carrier material. This may also be packaged in a squeeze bottle or in admixture with a pressurized volatile, normally gaseous propellant. The aerosol preparations can contain solvents, buffers, surfactants, and antioxidants in addition to the protein of the invention.

Other pharmaceutically acceptable carriers for the nucleic acid vaccine compositions according to the present invention are liposomes, pharmaceutical compositions in which the active protein is contained either dispersed or variously present in corpuscles consisting of aqueous concentric layers adherent to lipidic layers. The active protein is preferably present in the aqueous layer and in the lipidic layer, inside or outside, or, in any event, in the non-homogeneous system generally known as a liposomic suspension. The hydrophobic layer, or lipidic layer, generally, but not exclusively, comprises phospholipids such as lecithin and sphingomyelin, steroids such as cholesterol, more or less ionic surface active substances such as dicetylphosphate, stearylamine or phosphatidic acid, and/or other materials of a hydrophobic nature. Those skilled in the art will appreciate other suitable embodiments of the present liposomal formulations.

DELIVERY OF VACCINE NUCLEIC ACID TO CELLS AND ANIMALS

The following references set forth principles and current information in the field of basic, medical and veterinary virology and are incorporated by reference: *Fields Virology,* Fields, BN *et al,* eds., Lippincott Williams & Wilkins, NY, 1996; *Principles of Virology: Molecular Biology, Pathogenesis, and Control, Flint,* SJ. *et al,* eds., Amer Soc Microbiol, Washington DC, 1999; *Principles and Practice of Clinical Virology,* 4th Edition, Zuckerman. A.J. *et al,* eds, John Wiley & Sons, NY, 1999; *The Hepatitis C Viruses,* by Hagedorn, CH *et al,* eds., Springer Verlag, 1999; *Hepatitis B Virus: Molecular Mechanisms in Disease and Novel Strategies for Therapy,* Koshy, R. *et al,* eds, World Scientific Pub Co, 1998; *Veterinary Virology,* Murphy, F.A. *et al,* eds., Academic Press, NY, 1999; *Avian Viruses: Function and Controljtitchie,* B.W., Iowa State University Press, Ames , 2000; *Virus Taxonomy: Classification and Nomenclature of Viruses: Seventh Report of the International Committee on Taxonomy of Viruses,* by M. H. V. Van Regenmortel, MHV *et al,* eds., Academic Press; NY, 2000.

The Examples below describe certain preferred approaches to delivery of the vaccines and combinations of the present invention, intradermally by gene gun or intramuscularly.. A broader description of other approaches including viral and nonviral vectors and delivery mechanisms follow.

DNA delivery involves introduction of a "foreign" DNA into a cell *ex vivo* and ultimately, into a live animal or directly into the animal. Several general strategies for gene delivery (= delivery of nucleic acid vectors) for purposes that include "gene therapy" have been studied and reviewed extensively (Yang, N-S., *Crit. Rev. Biotechnol.* 72:335-356 (1992); Anderson, WF, *Science* 255:808-13, 1992; Miller, AS, *Nature* 357:455-60, 1992; Crystal, RG, *Amer. J. Med. 92(suppl* <54):44-52S, 1992; Zwiebel, JA *et al., Ann NYAcad Sc.* 575:394-404, 1991; McLachlin, JR *et al., Prog. Nucl. Acid Res. Molec. Biol.* 35:91-135, 1990; Kohn, DB *et al., Cancer Invest.* 7:179-92, 1989), which references are herein incorporated by reference in their entirety).

One approach comprises nucleic acid transfer into primary cells in culture followed by autologous transplantation of the *ex vivo* transformed cells into the host, either systemically or into a particular organ or tissue.

The term "systemic administration" refers to administration of a composition or agent such as a DNA vaccine as described herein, in a manner that results in the introduction of the composition into the subject's circulatory system or otherwise permits its spread throughout the body.

"Regional" administration refers to administration into a specific, and somewhat more limited, anatomical space, such as intraperitoneal, intrathecal, subdural, or to a specific organ.

"Local administration" refers to administration of a composition or drug into a limited, or circumscribed, anatomic space, such as intratumoral injection into a tumor mass, subcutaneous injections, intradermal or intramuscular injections

Those of skill in the art will understand that local administration or regional administration may also result in entry of a composition into the circulatory system - i.e., rendering it systemic to one degree or another.

For accomplishing the objectives of the present invention, nucleic acid therapy would be accomplished by direct transfer of a the functionally active DNA into mammalian somatic tissue or organ *in vivo*. DNA transfer can be achieved using a number of approaches described below. These systems can be tested for successful expression *in vitro* by use of a selectable marker *(e.g.,* G418 resistance) to select transfected clones expressing the DNA, followed by detection of the presence of the antigen-containing expression product (after treatment with the inducer in the case of an inducible system) using an antibody to the product in an appropriate immunoassay. Efficiency of the procedure, including DNA uptake, plasmid integration and stability of integrated plasmids, can be improved by

linearizing the plasmid DNA using known methods, and co-transfection using high molecular weight mammalian DNA as a "carrier".

The DNA molecules encoding the fusion polypeptides of the present invention may also be packaged into retrovirus vectors using packaging cell lines that produce replication-defective retroviruses, as is well-known in the art *(e.g.,* Cone, R.D. *et al, Proc Natl Acad Sci USA* §7:6349-53, 1984; Mann, RF *et al, Cell* 33:153-9, 1983; Miller, AD *et al, Molec Cell Biol* 5:431-7, 1985; Sorge, J, *et al, Molec Cell Biol 4:1130-1,* 1984; Hock, RA *et al, Nature 320:251,* 1986; Miller, AD *et al, Molec Cell Biol* 6:2895-2902 (1986). Newer packaging cell lines which are efficient an safe for gene transfer have also been described (Bank *et al,* US Pat. 5,278,056).

The above approach can be utilized in a site specific manner to deliver the retroviral vector to the tissue or organ of choice. Thus, for example, a catheter delivery system can be used (Nabel, EG *et al, Science 244:1342* (1989)). Such methods, using either a retroviral vector or a liposome vector, are particularly useful to deliver the nucleic acid to be expressed to a blood vessel wall, or into the blood circulation of a tumor.

Other virus vectors may also be used, including recombinant adenoviruses (Horowitz, MS, In: *Virology,* Fields, BN *et al,* eds, Raven Press, NY, 1990, p. 1679; Berkner, KL, *Biotechniques* (5:616-29, 1988; Strauss, SE, In: *The Adenoviruses,* Ginsberg, HS, ed., Plenum Press, NY, 1984, chapter 11), herpes simplex virus (HSV) for neuron-specific delivery and persistence. Advantages of adenovirus vectors for human gene delivery include the fact that recombination is rare, no human malignancies are known to be associated with such viruses, the adenovirus genome is double stranded DNA which can be manipulated to accept foreign genes of up to 7.5 kb in size, and live adenovirus is a safe human vaccine organisms. Adeno-associated virus is also useful for human therapy (Samulski, RJ *et al, EMBO J.* 70:3941, 1991) according to the present invention.

Another vector which can express the DNA molecule of the present invention, and is useful in the present therapeutic setting is vaccinia virus, which can be rendered non-replicating (U.S. Pats. 5,225,336; 5,204,243; 5,155,020; 4,769,330; Fuerst, TR *et al, Proc. Natl. Acad Sci. USA 86:2549-53,* 1992; Chakrabarti, S *et al, MoI Cell Biol* 5:3403-9, 1985). Descriptions of recombinant vaccinia viruses and other viruses containing heterologous DNA and their uses in immunization and DNA therapy are reviewed in: Moss, B, *Curr Opin Genet Dev* 3:86-90, 1993; Moss, B, *Biotechnol 20:345-62,* 1992);.

In addition to naked DNA or viral vectors, engineered bacteria may be used as vectors. A number of bacterial strains including *Salmonella,* BCG and *Listeria monocytogenes(LM)* (Hoiseth *et al, Nature* 297:238-9, 1981 ; Poirier, TP *et al, J Exp Med 168:25-32,* 1988); Sadoff, JC *et al, Science 240:336-%,* 1988; Stover, CK *et al, Nature* 357:456-60, 1991; Aldovini, A *et al, Nature* 357:479-82, 1991). These organisms display two promising characteristics for use as vaccine vectors: (1) enteric

routes of infection, providing the possibility of oral vaccine delivery; and (2) infection of monocytes/macrophages thereby targeting antigens to professional APCs.

In addition to virus-mediated gene transfer *in vivo,* physical means well-known in the art can be used for direct transfer of DNA, including administration of plasmid DNA (Wolff *et at,* 1990, *supra)* and particle-bombardment mediated gene transfer (Yang, N-S, *et at, Proc Natl Acad Sci USA 87:956%,* 1990; Williams, RS *et at, Proc Natl Acad Sci USA 88:2726,* 1991; Zelenin, AV *et at, FEBS Lett 280:94* , 1991; Zelenin, AV *et at, FEBS Lett 244:65,* 1989); Johnston, SA *et at, In Vitro Cell Dev Biol* 27:1 1, 1991). Furthermore, electroporation, a well-known means to transfer genes into cell *in vitro,* can be used to transfer DNA molecules according to the present invention to tissues *in vivo* (Titomirov, AV *etat, Biochim Biophys Acta* 7055:131, 1991).

"Carrier mediated gene transfer" has also been described (Wu, CH *et at, J Biol Chem 264:\69%5,* 1989; Wu, GY *et at, J Biol Chem 263:14621,* 1988; Soriano, P *et at, Proc Nat. Acad Sci USA* 50:7128, 1983; Wang, C-Y *et at, Pro. Natl Acad Sci USA* 54:7851, 1982; Wilson, JM *et at, J Biol Chem 267:963,* 1992). Preferred carriers are targeted liposomes (Nicolau, C *et at, Proc Natl Acad Sci USA* 50:1068, 1983; Soriano *et at, supra)* such as immunoliposomes, which can incorporate acylated mAbs into the lipid bilayer (Wang *et at, supra).* Polycations such as asialoglycoprotein/poly lysine (Wu *et at,* 1989, *supra)* may be used, where the conjugate includes a target tissue-recognizing molecule *{e.g.,* asialo-orosomucoid for liver) and a DNA binding compound to bind to the DNA to be transfected without causing damage, such as polylysine. This conjugate is then complexed with plasmid DNA of the present invention.

Plasmid DNA used for transfection or microinjection may be prepared using methods well-known in the art, for example using the Quiagen procedure (Quiagen), followed by DNA purification using known methods, such as the methods exemplified herein.

EXAMPLE 1

MATERIALS AND METHODS

Plasmid DNA Construction

The generation of pcDNA3, pcDNA3-E7, pcDNA3-CRT/E7 (Cheng *et at, supra),* pcDNA3-E7/HSP70 (Chen CH *et at, Cancer Res 60:* 1035-42, 2000), and pcDNA3-ETA(dII)/E7 (Hung CF *et at, Cancer Res* 2001; 61:3698-3703) has been described previously. To generate pcDNA3-Sig/E7/LAMP-l, Sig/E7/LAMP-1 was cut at the EcoRI/BamHI sites from pCMV(neo)-Sig/E7/LAMP-l (Ji H *et at, Hum Gene Ther* 70:2727-40, 1999) and cloned into pcDNA3.

For generation of pNGVL4a-E7(detox), the E7 gene was cloned into pNGVL4a (National Gene Vector Laboratory) using the *EcoRI* and *Kpnl* restriction sites. Using site-directed mutagenesis, two

point mutations, which had previously been found to reduce Rb binding (Munger K *et at, EMBO J* 5:4099-4105, 1989), were introduced into the E7 gene. The primers used to introduce these mutations were as follows:

E7(detox) Forward: 5' ctgatctctacggttatgggcaattaaatgacagctc 3' (SEQ ID NO:21) and

E7(detox) Reverse: 5' gagctgtcatttaattgcccataaccgtagagatca 3' (SEQ ID NO:22).

For generation of pNGVL4a-CRT/E7(detox), CRT was PCR amplified with the following primers

5' aaagtcgacatgctgctatccgtgccgctgc 3' (SEQ ID NO:23) and

5' gaattcgttgtctggccgcacaatca 3' (SEQ ID NO:24).

using a human CRT plasmid as a template (which was kindly provided by Dr. David Llewellyn of Dept. Medical Biochemistry at University of Wales College of Medicine at Cardiff, U.K.). The PCR product was cut with Sal I/EcoRI and cloned into the Sal I/EcoRI sites of pNGVL4a-E7(detox). The accuracy of DNA constructs was confirmed by DNA sequencing.

For the generation of pcDNA3-N-CRT, DNA encoding the N-domain of CRT, N-CRT was first amplified with PCR by using rabbit CRT cDNA as the template (Michalak, M., *et at, Biochem Cell Biol* 7d;779-85, 1998) and a set of primers:

5'-ccggtctagaacgctgctccctgtgccgct-3' [SEQ ID NO:25] and

5'-cccgaattcgttgtccgggccgcacgatca-3' [SEQ ID NO:26].

The amplified product was further cloned into the Xbal/EcoRl site of pcDNA3 (Invitrogen Corp)

For the generation of pcDNA3-P-CRT, DNA encoding the P-domain of CRT was first amplified with PCR using rabbit CRT cDNA as the template and a set of primers,

5'-tgctctagaatgtacaagggtgagtggaagcc-3' [SEQ ID NO:27] and

5'-ccggaattccagctcgtccttggcctggcc-3' [SEQ ID NO:28].

The amplified product was further cloned into the Xbal/EcoRI site of pcDNA3.

For the generation of pcDNA3-C-CRT, DNA encoding the C-domain of CRT was first amplified with PCR using rabbit CRT cDNA as the template and a set of primers,

5'-tgctctagaatgtacaagggtgagtggaagc-3' [SEQ ID NO:29] and

5'-ccggaattccagctcgtccttggcctggc-3'. [SEQ ID NO:30].

The completed product was then cloned into the Xbal/EcoRI site of pcDNA3.

For the generation of pcDNA3-N-CRT/E7, P-CRT/E7, and C-CRT/E7, DNA encoding E7 was first amplified with pcDNA3-E7 as a template and a set of primers,

5'-ggggaattcatggagatacaccta-3' [SEQ IDNO:31] and

5'-ggtggatccttgagaacagatgg-3' [SEQ ID NO:32],

and then cloned into the EcoRI/BamHI sites of pcDNA3-N-CRT, pcDNA3-P-CRT, or pcDNA3-C-CRT to generate pcDNA3-N-CRT/E7, pcDNA3-P-CRT/E7, or pcDNA3-C-CRT/E7.

Mice

Six- to eight-week-old female C57BL/6 mice were purchased from the National Cancer Institute (Frederick, Maryland) and kept in the oncology animal facility of the Johns Hopkins Hospital (Baltimore, Maryland). IFN-γ knockout mice were obtained from the Jackson Laboratory (Bar Harbor, Maine). All animal procedures were performed according to approved protocols and in accordance with the recommendations for the proper use and care of laboratory animals.

Generation of TC-I and TC-I P3 (A15) Tumor Cell Line

The production and maintenance of TC-I cells has been described by Lin *et al. supra.*

For the generation of TC-I P3 (A15), Vac-Sig/E7/LAMP-1 -vaccinated mice were challenged with TC-I tumor cells. Vaccination with Vac-Sig/E7/LAMP-1 elicits E7-specific antitumor responses against HPV-16 E7-expressing tumors (TC-I), although the vaccine fails to prevent tumor formation in approximately 20% of the vaccinated mice (Lin *et al., supra).* The outgrown TC-I tumors from Vac-Sig/E7/LAMP-1 vaccinated mice were explanted, cut into pieces of less than 1 mm in diameter, digested with collagenase at a concentration of 1 mg/ml in DMEM (GIBCO BRL, Rockville, MD), and expanded *in vitro.* These expanded cell lines were called TC-I Pl. Vac-Sig/E7/LAMP-1 vaccinated mice were then challenged with TC-I Pl tumor cells. Approximately 40 % of vaccinated mice developed tumors (not shown). The outgrown tumors from these vaccinated mice were then explanted and expanded *in vitro* to create the TC-I P2 tumor cell line. Vac-Sig/E7/LAMP-1 vaccinated mice were then challenged with TC-I P2 tumor cells. This time approximately 60-80% of vaccinated mice developed tumors (not shown). The outgrown tumors from these vaccinated mice were further explanted and expanded *in vitro* to generate the TC-I P3 tumor cell line. 50 TC-I P3 clones were generated by limiting dilution. Among the TC-I P3 clones, a representative clone with marked down-regulation of MHC class I expression was isolated and expanded, creating the TC-I P3 (Al 5) tumor cell line. We have determined that more than 90% of the TC-I P3 (Al 5) cells exhibited down-regulated MHC class I expression. Less than 10% of the TC-I P3 (Al 5) cells expressed any MHC class I molecules. Monoclonal antibody (mAb) Anti-H-$2K^b$/H-$2D^b$ (clone 28-8-6) (BD Bioscience, San Diego, CA) was used to detect MHC class I expression. Both TC-I and TC-I P3 (Al 5) cells were grown in RPMI 1640, supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin/ streptomycin, 2mM L-glutamine, ImM sodium pyruvate, 2mM nonessential amino acids, and 0.4 mg/ml G41 8 at 37^0C with 5% CO_2. On the day of tumor challenge, tumor cells were harvested by trypsinization, washed twice with IX Hanks buffered salt solution (HBSS), and resuspended in IX HBSS to the designated concentration for injection.

DNA Vaccination

For the gene gun mediated intradermal vaccination, DNA-coated gold particles (1 μg DNA/bullet) were delivered to the shaved abdominal region of C57BL/6 mice using a helium-driven gene gun (BioRad, Hercules, CA) with a discharge pressure of 400 p.s.i., as previously described (Chen CH *et al. Cancer Res* 60: 1035-42, 2000). C57BL/6 Mice were vaccinated via gene gun with either 2 μg of pcDNA3, pcDNA3-E7, pcDNA3-CRT/E7, pcDNA3-E7/HSP70, pcDNA3-ETA(dII)/E7, pcDNA3-Sig/E7/LAMP-1, pNGVL4a, pNGVL4a- E7(detox), or pNGVL4a-CRT/E7(detox). These mice received a booster with the same regimen one week later.

For the intramuscular (i.m.) DNA vaccination, 50μg/mouse of pNGVL4a, pNGVL4a- E7(detox), and pNGVL4a-CRT/E7 (detox) DNA vaccines were delivered i.m. by syringe needle injection. These mice received a booster with the same regimen one week later.

Intracellular Cytokine Staining and Flow Cytometry Analysis

Staining of cell surface CD8 and intracellular EFN-γ as well as FACScan analysis was performed as described previously (Chen *et al, supra)* Prior to FACScan, spleen cells from different groups of mice were collected and incubated for 20 hours with 1 μg/ml of E7 peptide (aa 49-57, RAHYNIVTF, SEQ DD NO:33) (Feltkamp MC *et al, Eur J Immunol 23:2242-49,* 1993) containing an MHC class I epitope for detecting E7-specific CD8+ T cell precursors. Golgistop (Brefeldin A) was added 6 hours before harvesting the cells from the culture. Triplicate experiments were performed using a pool of spleen cells from the group of mice vaccinated with the same vaccine construct. The number of IFN-γ-secreting CDS^+ T cells was analyzed using flow cytometry. Analysis was performed on a Becton-Dickinson FACScan with CELLQuest software (Becton Dickinson Immunocytometry System, Mountain View, CA).

In Vivo Tumor Therapy fusing TC-I tumors)

For *in vivo* tumor therapy experiments using an E7-expressing tumor (TC-I), mice (5/group) were challenged intravenously (iv) through the tail vein with 10^4 TC-I cells. Seven days after tumor challenge, mice were administered 2 μg of various DNA vaccines or control plasmids via gene gun. One week after the first vaccination, the mice were boosted with the same regimen. Mice were sacrificed and lungs explanted on day 28. The pulmonary nodules on the surface of the lungs of each mouse were counted by experimenters blinded to sample identity as described previously (Ji *et al, supra).*

Long Term (8 week) In Vivo Tumor Protection Experiment

Mice (5/group) were vaccinated via gene gun with 2 μg of various DNA vaccines or control plasmids. One week later, mice were boosted with the same regimen. Eight weeks after the initial vaccination, mice were challenged iv with 10^5 TC-I cells via tail vein. Mice were sacrificed 28 days

after the tumor challenge and lung surface pulmonary nodules in each mouse were counted by experimenters blinded to sample identity.

In Vivo Tumor Protection against TC-I P3 (A15) Class I Down-regulated Tumors.

C57BL/6 mice (5/group) were vaccinated with various DNA vaccines or control plasmids as described below by gene gun injection. One week after the last vaccination, mice were challenged with $5x10^4$ TC-I P3 (Al 5) tumor cells by subcutaneous (s.c.) injection in the right leg. Tumor growth was monitored by visual inspection and palpation twice weekly as described previously (Lin *et al, supra).*

For studies comparing wild type and IFN-γ KO C57BL/6 mice and mice, mice (5/group) were vaccinated via gene gun with 2 μg of pcDNA3-CRT/E7 DNA and then boosted with the same regimen one week later. One week after the last vaccination, mice were challenged with $5x10^4$ TC-I P3 (A 15) tumor cells sc in the right leg. Tumor growth was monitored by visual inspection and palpation twice weekly.

Immunohistochemical Labeling for the Quantitation of Microvessel Density

Labeling of intratumoral microvessels was performed with rat anti-mouse CD31 mAb (1:30 dilution, Bioscience), followed by VECTOR® M.O.M Immunodetection Kit (VECTOR; Burlingame, CA) using a known method (Cheng, WF *et al, Cancer* #5:651-7, 1999). In each section, the three most vascularized areas were selected and microvessels counted at 200X magnification; the mean MVD count of the three fields for each tumor was calculated count. Large vessels with thick muscular walls and lumina with diameters accommodating >~8 blood cells were excluded. MVDs were evaluated in tumors of similar size. All measurements were performed by a single pathologist, blinded to sample identity.

In Vivo Angiogenesis Assay using Matrigel®

In vivo angiogenesis was assessed using the Matrigel® plug assay, basically according to Cheng *et al,* 2001, *supra* and Coughlin, CM *et al., JClin Invest* 707:1441-52, 1998). Mice were immunized with 16 μg of plasmid DNA lacking any insert (negative control), or with wild-type E7, N-CRT, N-CRT/E7, P-CRT/E7, C-CRT/E7 or CRT/E7 DNA on day 0 and were boosted with the same regimen on day 7. Matrigel® (Becton Dickinson and Co., Franklin Lakes, NJ) was mixed with heparin (final concentration of 50 U/ml), bFGF (final concentration of 20 ng/ml), and VEGF (final concentration of 200 ng/ml) at 4°C. A total of 0.5 ml of this Matrigel® mixture was injected sc into the abdominal midline of DNA-vaccinated mice on day 7. Naive mice injected with Matrigel® mixed with heparin, bFGF and VEGF served as a positive control; naive mice injected with Matrigel® alone were negative controls. Mice were euthanized on day 16. The Matrigel® plugs were resected from surrounding connective tissues. Half of the plugs were fixed in 10% formaldehyde, embedded in paraffin, sectioned, and stained with hematoxylin and eosin or Giemsa stains to calculate MVD. In each section, the five most vascular areas were selected, microvessel were evaluated at 400X magnification, and the mean

number in the five fields for the Matrigel® plugs was calculated (MVD count). The remaining half of each plug was assayed for hemoglobin content according to manufacturer's instructions (Drabkin's reagent kit; Sigma Diagnostics Co., St. Louis, MO).

Statistical Analysis

All results are expressed as means ± SE and are representative of at least two different experiments. Data for ICCS with flow cytometric analysis and tumor treatment experiments were analyzed by analysis of variance (ANOVA). Comparisons between individual data points were made using Student's t-test. Kaplan-Meier survival curves were used for tumor protection experiments for differences between curves, and/? values were calculated using the log-rank test. /?<0.05 was considered significant.

EXAMPLE 2

Comparative Analysis of CRT/E7 DNA Vaccine with other IPP's Linked to E7

CD8+ T cell-mediated immune responses are important in controlling both HPV infections and HPV-associated neoplasms. To assess immune response to various DNA vectors, the frequency of E7-specific $CD8^+$ T cell precursors generated by pcDNA3, pcDNA3-E7, pcDNA3-CRT/E7, pcDNA3-E7/HSP70, pcDNA3-ETA(dII)/E7, and pcDNA3-Sig/E7/LAMP-1 vaccine constructs, ICCS with flow cytometric analysis was done using spleen cells from vaccinated mice one week after the last vaccination. As shown in **Figures 1 and 2,** mice vaccinated with pcDNA3-CRT/E7 DNA exhibited the highest numbers of E7-specific IFN-γ^+ $CD8^+$ T cell precursors (per $3xlO^5$ spleen cells) - 655 - compared to mice vaccinated with pcDNA3-E7/HSP70, pcDNA3-ETA(dII)/E7, pcDNA3-Sig/E7/LAMP-1, pcDNA3-E7, or pcDNA3 ($p<0.05$).

EXAMPLE 3

Mice Immunized with CRT/E7 Vaccine Generate Potent Antitumor Responses

Therapeutic potential of the various chimeric DNA constructs were tested for treatment of an E7-expressing tumor, TC-I, using a previously described lung hematogenous spread model (Ji *et al, supra).* As shown in **Figure** 3, mice given the pcDNA3-CRT/E7 vaccine exhibited significantly lower numbers of pulmonary nodules compared to mice vaccinated with pcDNA3 (negative control) or pcDNA3-E7 after TC-I challenge O<0.05). When comparing pcDNA3-CRT/E7 to pcDNA3-E7/HSP70, pcDNA3-ETA(dII)/E7, or pcDNA3-Sig/E7/LAMP-1 vaccines, pcDNA3-CRT/E7-immunized mice displayed lower mean numbers of pulmonary nodules than the others($p<0.95$).

EXAMPLE 4

Vaccination with CRT/E7 Generates Higher Numbers of Antigen- -Specific Memory T Cells and Provides Long-Term Tumor Protection

To measure E7-specific $CD8^+$ memory T cell precursors generated by various DNA vaccines, animals were vaccinated with pcDNA3 (negative control), pcDNA3-E7 (antigen control), pcDNA3-CRT/E7, pcDNA3-E7/HSP70, pcDNA3-ETA(dIT)/E7, and pcDNA3-Sig/E7/LAMP-l DNA. ICCS flow cytometric were performed on spleen cells derived from vaccinated mice eight weeks after the initial vaccination. As shown in Figures 4 & 5, mice pcDNA3-CRT/E7 DNA induced the highest number of E7-specific IFN-γ^+ $CD8^+$ memoiy T cell precursors compared to mice vaccinated with the other DNA constructs ($p<0.05$).

This vaccine also provided the best long-term protection against E7-expressing tumors. A long-term tumor protection study was done comparing even a larger number of IPP-Ag vaccine constructs: pcDNA3 (neg. control), pcDNA3-E7 (antigen control), pcDNA3-CRT/E7, pcDNA3-E7/HSP70, pcDNA3-VP22/E7 (Herpes simplex virus protein VP22) , pcDNA3-ETA(dII)/E7, and pcDNA3-Sig/E7/LAMP-1 DNA. As shown in **Figure** 6, mice vaccinated with the pcDNA3-CRT/E7 DNA exhibited significantly fewer pulmonary nodules compared to mice vaccinated with pcDNA3-Sig/E7/LAMP-1, pcDNA3-E7, or pcDNA3 ($p<0.05$). Although not statistically significant, the pcDNA3-CRT/E7 vaccinated mice also displayed lower mean numbers of pulmonary nodules than mice vaccinated with pcDNA3-E7/HSP70, pcDNA3-VP22/E7, pcDNA3-ETA(dII)/E7 ($p<0.70$).

EXAMPLE 5

Vaccination with CRT/E7 DNA Results in Immunity that Controls E7-Expressing Tumors in which MHC Class I was Down-regulated

Since MHC class I expression is down-regulated in most cervical cancers, it was important to determine the effectiveness of the CRT/E7 DNA vaccines in protecting against tumors with low MHC class I expression. An E7-expressing murine tumor model with down-regulated MHC class I expression, termed TC-I P3 (Al 5), was developed as described in Example 1. MHC class I expression in TC-I PO and TC-I P3 (Al 5) tumor cells was done by flow cytometric analysis. As shown in **Figure** 7, MHC class **I** expression was markedly down-regulated in TC-I P3 (Al 5) compared to TC-I.

This was followed by a tumor protection study in C57BL/6 mice in which mice were vaccinated with 2 μg of CRT/E7 DNA, CRT DNA (CRT-only control) , E7 DNA (antigen control), or vector alone (negative control), followed by challenge one week later with $5x10^4$ TC-I P3 (Al 5) tumor cells. As shown in **Figure** 8, all mice vaccinated with pcDNA3-CRT/E7 DNA were protected against tumor challenge with TC-I P3 (A15) for a period of up to 45 days after tumor challenge. In contrast, all

control mice (vaccinated with pcDNA3-CRT, pcDNA3-E7, or pcDNA3 vector) developed tumors within 14 days.

The present inventors and colleagues had previously shown that IFN-γ was essential for an anti¬ tumor effect generated by DNA vaccines employing *Mycobacterium tuberculosis* HSP70 linked to E7 (against an E7-expressing tumor cell line with down-regulated MHC class I (Cheng WF *et al Gene Ther 10:* 131 1-20, 2003. To determine whether if IFN-γ was also essential in the case of vaccination with CRT/E7 DNA, an experiment was done wherein wild type and IFN-γ KO mice were vaccinated and challenged with TC-I P3 (Al 5). As shown in **Figure** 9, whereas 100% of wild-type mice vaccinated with CRT/E7 were protected against challenge with TC-I P3 (Al 5), only 20% of IFN-γ KO mice so vaccinated E7 were protected.

These foregoing results indicate that (a) immunization with CRT/E7 DNA can control E7-expressing tumors with down-regulated MHC class I expression and (b) that this protection is dependent upon the ability to make, express or upregulate IFN-γ (presumably in T cells).

EXAMPLE 6

Intramuscular and Gene Gun Immunization with pNGVL4a-CRT/E7(detox) DNA Enhances E7-specific CD8+ T Cell Immune Responses

Because the pcDNA3-CRT/E7 DNA vector contains ampicillin resistance gene and the wild type E7 has a greater risk of oncogenically transforming host cells, a different vector more suitable for human use was employed. The present inventors used the pNGVL4a vector (see, for example, the present inventors' patent publication WO 2004/098526A2) to create a new CRT/E7 vector designated "pNGVL4a-CRT/E7(detox)" for use in the clinical setting. Moreover, since it is likely that human immunization will be performed by i.m. injection, a test was conducted to demonstrate that i.m. vaccination with pNGVL4a-CRT/E7(detox) in mice can enhance E7-specific CD8+ T cell immune responses. As shown in **Figures 10 and** 11, i.m. and gene gun immunization of mice with pNGVL4a-CRT/E7(detox) DNA significantly increased the number of E7-specific CD8+ T cell precursors compared to vaccination with an antigen control (pNGYL4a-E7 (detox)) or negative vector control (pNGVL4a vector only) ($p<0.05$).

Gene gun-mediated immunization with pNGVL4a-CRT/E7(detox) generated more antigen-specific CD8+ T cell precursors than did i.m. immunization ($p<0.05$).

Furthermore, in the tumor protection experiments using E7-expressing tumors with down-regulated MHC class I expression (TC-I P3 (Al 5)), the results indicated that mice vaccinated with pNGVL4a-CRT/E7(detox) DNA demonstrated 100% protection against TC-I P3 (Al 5) up to 45 days after tumor challenge (not shown).

These results indicate that the pNGVL4a-CRT/E7(detox) DNA, like the pcDNA3-CRT/E7 DNA, is capable of markedly potentiating the number of E7-specific CD8+ T cells and resulting in a state in which E7-expressing tumors, even with down-regulated MHC class I expression, are controlled by the immune system. Thus, pNGVL4a-CRT/E7(detox) DNA is a useful vaccine construct for use in humans for control of HPV infections and HPV associated lesions.

EXAMPLE 7

Discussion of Examples 1-6

E7-specific CD8+ T cell immune responses and anti-tumor effects were compared across generated by five effective chimeric DNA vaccines in which DNA encoded a fusion protein of the antigen and an IPP. The chimeric combinations tested were: CRT/E7, E7/HSP70, ETA(dII)/E7, Sig/E7/LAMP-1/E7) that the inventors' laboratory had previously developed

Mice vaccinated with pcDNA3-CRT/E7 construct generated the highest quantity of antigen-specific CD8+ T cell precursors and memory T cells, resulting in potent tumor therapeutic and long-term tumor protective effects.

The potentiated immune response induced by the CRT/E7 DNA vaccine could even control tumors with down-regulated MHC class I expression. This indicates that the vaccine would be useful for treating patients with cervical lesions in whom MHC class I expression was also down-regulated, a condition which normally facilitates evasion of a CD8+ CTL response by the tumor.

Also demonstrated herein is a pNGVL4a-CRT/E7(detox) DNA vaccine that has several advantages for clinical use over the pcDNA3 vaccine discussed above. The pNGVL4a-CRT/E7(detox) DNA vaccine, administrated via gene gun or intramuscularly, was able to potentiate the E7-specific $CD8^+$ T cell immune response and control tumors with down-regulated class I.

Indeed, one concern about efficacy of immunotherapy is that tumors can evade immune responses through various mechanisms, including MHC class I down-regulation. A number of human cancers have been shown to downregulate MHC class I expression, including melanoma (Ferrone S *et al, Immunol Today* 7(5:487-94, 1995); lung cancer (Korkolopoulou P *et al, Br J Cancer* 73:148-53, 1996), prostate cancer (Sanda MG *et al. J Natl Cancer Inst* <57:280-5, 1995), breast cancer (Cabrera T *et al.* High frequency of altered HLA class I phenotypes in invasive breast carcinomas. *Hum Immunol* 50:127-34, 1996; Vitale M *et al, Cancer Res;* 58: 737-42, 1998), ovarian and colon cancer (Vegh Z *et al, Cancer Res* 53:2416-20, 1993) and cervical cancer (Connor ME *et al., M J Cancer* 4(5:1029-34, 1990; Cromme FV *et al. Oncogene* 5:2969-75, 1993; Koopman LA *et al J Exp Med 191:* 961-76, 2000).

By down-regulating MHC class I expression, tumor cells escape immune recognition; thus avoid killing by antigen-specific $CD8^+$ T cells. The present invention provides a composition and method - using a CRT/E7 DNA vaccine against tumors with low MHC class I expression, - that generates a stronger immune response that is also able to overcome such evasion and thus be used for treating patients with advanced cervical cancer in which MHC class I expression is down-regulated.

Based on the present inventors previous studies with E7/HSP70, it was concluded that IFN-γ was required for tumor protection. IFN-γ could indeed up-regulate MHC class I expression on TC-I P3 (Al 5) tumor cells to levels equivalent to "wild type" TC-I PO cells. The present discovery that CRT/E7 DNA vaccine required IFNγ for its immunopotentiating and anti-tumor action further supports a role for the stimulation of IFNγ expression (or an increase in the number of CD8+ T cells expressing cytoplasmic IFNγ in an anti-tumor immune response against tumor cells in which MHC class I expression was downregulated.

Tests of a novel immunogenic vector, pNGVL4a-CRT/E7(detox), showed that successful immunization could be achieved by both the i.m. route and by i.d. gene gun administration, leading to both an increase in the numbers of . $CD8^+$ T cells and efficacy against a tumor in which MHC class I is down-regulated. Intradermal immunization via gene gun directly targets antigen to professional APCs, Langerhans cells, allowing the intracellular strategy to further improve direct presentation of antigen to T cells by DNA-transfected DCs. In comparison, intramuscular immunization likely targets antigen to myocytes, and the antigen encoded by DNA vaccine is eventually presented through bone marrow-derived APCs through the cross-priming mechanism. Vaccination with pNGVL4a-CRT/E7(detox) likely led to secretion of chimeric CRT/E7(detox) protein or lysis of cell expressing CRT/E7(detox) antigen, releasing the chimeric protein from cells to be taken up and processed by nearby APCs via the MHC class I-restricted pathway. The linkage of CRT to E7 facilitates cross-priming of E7 antigen. One recent study found that CD91, an α2 macroglobulin receptor commonly expressed on professional APCs, serves as a receptor for HSPs and CRT, and facilitates cross-priming (Basu S *et al, Immunity* /4:303-13, 2001). Intramuscular immunization of DNA vaccines encoding CRT/E7(detox) may promote prolonged release of the CRT/E7(detox) protein from transfected cells to target, concentrate CRT/E7(detox) to professional APCs, and facilitate the cross-priming of an E7-specific response. Therefore different routes of administration may generate different degrees of immune responsivity by the same vaccine.

In summary, CRT/E7 DNA vaccines are attractive therapeutic vaccines not only because of the ability to generate effective anti-tumor immunity against E7-expressing tumors and to control E7-expressing tumors with down-regulated MHC class I expression in vaccinated mice, but also because of the ability to prevent or delay tumor growth by targeting tumor vasculature.

It should be evident that the utility of a CRT/E7 DNA vaccine (particularly using E7(detox) is not limited to treating patients with E7-expressing cervical cancers but is also useful to treat subjects who are in need of anti-angiogenic therapy that is mediated by CRT independent of the antigen with which it is linked. The same is true of a related vaccine employing CRT/E6, or any other antigen for that matter.

EXAMPLE 8

DNA Vaccines Encoding CRT Domains Elicit Potent T cell-mediate Immune Responses Anti-tumor Immunity and Inhibition of Angiogenesis

(This Example specifically incorporates by reference Cheng et ah, Vaccine 23 3864-3874, 2005)

Vaccination with DNA encoding N-CRT, P-CRT, or C-CRT linked to E7 significantly **enhances the E7-specific CD8+ T cell response.** To determine if the different domains of calreticulin when linked with the E7 DNA vaccines could enhance E7-specific T cell-mediated immune responses in mice, we performed ICCS with flow cytometric analysis to characterize E7-specific CDS^+ and $CD4^+$ T cell precursors generated as a result of immunization with CRT/E7 DNA or DNA encoding CRT domains linked to E7. Vaccination with each of N-CRT/E7, P-CRT/E7, and C-CRT/E7 DNA generated higher frequencies of E7-specific IFN-γ-secreting $CD8^+$ T cell precursors when compared to vaccination with E7 DNA *(p<0.0\)*. Physical linkage of N-CRT to E7 was required for this enhancement since administration of a mixture of DNA encoding the N- CRT and E7 DNA did not enhance $CD8^+$ T cell activity. Vaccination with CRT/E7 DNA generated a slightly higher number of E7-specific $CD8^+$ T cell precursors (220.5±18.5) when compared to the N-CRT/E7 (178.0±18.5), P-CRT/E7 (140.0±16.0) and C-CRT/E7 (128.0±10.0) ($p<0.01$). Thus DNA encoding each CRT domain has immunopotentiating activity, and can serve as an IPP when linked to DNA encoding antigen. However, none of CRT domains linked to E7 stimulated more E7-specific IFN-γ-secreting $CD4^+$ T cells (compared to vaccination with E7 DNA).

Vaccination with N-CRT/E7 DNA significantly **enhanced the E7-specific** antibody **response.** Vaccination with CRT/E7 or N-CRT/E7, but not with P-CRT/E7 nor C-CRT/E7 DNA enhanced E7-specific antibody responses in vaccinated mice compared to vaccination with wild type E7 DNA ($p<0.01$). There was no significant difference between the titers of anti-E7 antibodies induced by N-CRT/E7 and vs. CRT/E7

Vaccination with N-CRT/E7, **P-CRT/E7 or C-CRT/E7 DNA enhanced** E7-specific **Tumor Protection.** *In vivo* tumor protection experiment were carried out as above using TC-I tumor cells. 100% of mice receiving N-CRT/E7, P-CRT/E7, C-CRT/E7, or CRT/E7 DNA remained tumor-free 60 days after TC-I challenge. In comparison, all mice vaccinated with wild-type E7 DNA only developed

tumors within 14 days of challenge. Thus, each domain of CRT can protect vaccinated mice against a lethal challenge with E7-expressing tumor cells when linked to the E7 antigen in a DNA vaccine.

Treatment with N-CRT/E7, P-CRT/E7, or C-CRT/E7 DNA Significantly **Reduced** Pulmonary **Tumor Nodules in C57BL/6 Mice.** The therapeutic potential of the above vaccines was tested using a the lung hematogenous spread model described above. C57BL/6 mice treated with N-CRT/E7 DNA (1.0±0.4), P-CRT/E7 (1.2±0.8), or C-CRT/E7 (1.4±0.6) all exhibited significantly fewer pulmonary tumor nodules than did mice treated with "control" DNA vaccines (a) wild-type E7 (antigen only) (139.0 ± 11.0) or (b) "no antigen" N-CRT (34.0±3.2) by one-way ANOVA ($p<0.001$). Thus DNA encoding each of the 3 CRT domains when linked with E7 DNA generated potentiated antitumor effects in a lung hematogenous spread model.

Treatment of mice with N-CRT DNA also resulted in significantly fewer tumor nodules than treatment with wild-type E7 DNA or no treatment (one-way ANOVA, $p < 0.001$), suggesting that N-CRT is capable of inducing non-immunological antitumor effects .

Treatment with N-CRT/E7 DNA or N-CRT DNA Significant Reduced Pulmonary Tumor Nodules in Immunocompromised Mice. To study this apparently T cell-independent (likely non-immunological) antitumor effect, immunocompromised BALB/c athymic nude mice *(nu/nu)* given N-CRT, N-CRT/E7, or CRT/E7 DNA displayed a significantly lower mean number of pulmonary tumor nodules (18.0 ± 2.0 for N-CRT, 25.0± 4.0 for N-CRT/E7) compared with mice treated with wild type E7 DNA (215.0 ± 10.0), plasmid without insert (217.5 ± 17.0), or untreated naive mice (230.0 ± 22.5) (one-way ANOVA, p< 0.001). Interestingly, neither P-CRT/E7 nor C-CRT/E7 stimulated significant reduction of tumor nodules compared to mice treated with wild-type E7 DNA. In addition, nude mice treated with N-CRT/E7 DNA exhibited significantly fewer pulmonary tumor nodules than nude mice treated with CRT/E7 DNA (one-way ANOVA, $p<0.05$). Thus, treatment with N-CRT, N-CRT/E7, or CRT/E7 DNA can generate an antitumor effects even in the absence of T cell-mediated immune responses, whereas P-CRT/E7 and C-CRT/E7 DNA did not have this effect.

CRT/E7, N-CRT/E7 and N-CRT Reduced Microvessel Density **(MVD) in Tumors of BALB/c nude Mice.** To evaluated the possibility that inhibition of angiogenesis could explain the above non-immunologic anti-tumor effects, MVD was evaluated in the pulmonary tumors of nude mice treated with various DNA vaccines. Treatment of nude mice with N-CRT, N-CRT/E7, or CRT/E7 DNA significantly reduced MVD in pulmonary tumors than did treatment with wild-type E7, P-CRT/E7 or C-CRT/E7 group (one-way ANOVA, *$p<0.001$).* N-CRT/E7 DNA treatment was more potent than CRT/E7 DNA (one-way ANOVA, *$p<0.05$).* Taken together, the results demonstrate that T cell-independent antitumor

effects elicited by vaccination with N-CRT, N-CRT/E7, or CRT/E7 DNA occurs via inhibition of tumor angiogenesis, an action that was associated in particular with the N-domain of CRT.

Reduced MVD and Hemoglobin (Hb) in Matrigel® Plugs from Mice Challenged with TC-I tumor Cells and treated with N-CRT, N-CRT/E7, or CRT/E7 DNA. A more quantitative assessment of antiangiogenesis in mice treated with the above DNA constructs, were performed in a Matrigel®-based *in vivo* angiogenesis assays. The Hb content of Matrigel implants from N-CRT, N-CRT/E7, or CRT/E7-treated mice were significantly lower than those from mice treated with insertless control DNA, E7 DNA, P-CRT/E7 DNA, or C-CRT/E7 DNA ($p<0.01$, ANOVA). This assay revealed that N-CRT or N-CRT/E7 DNA inhibited bFGF- or VEGF-induced *in vivo* angiogenesis. The Hb levels Matrigel® implants from N-CRT/E7-treated mice were significantly lower than those from CRT/E7-treated mice ($p<0.01$, ANOVA).

The MVD in these Matrigel® samples serve as an additional measure of angiogenesis inhibition, since angiogenesis permits red blood cells (source of Hb) to extravasate from vessels. The mean MVDs in Matrigel® samples from N-CRT (23.7 ± 10.4), N-CRT/E7 (21.3 ± 4.7), and CRT/E7 (29.0 ± 9.2) DNA-treated mice were similar to one another , but significantly lower than the samples from mice treated with control plasmid DNA (no insert) (98.3 ± 31.8), wild-type E7 DNA (76.7 ± 12.0), C-CRT/E7 DNA (77.3 ± 9.6), or P-CRT/E7 DNA (76.3 ± 6.7). This confirms that the N domain of CRT is responsible for the observed anti-angiogenic effects.

N-CRT DNA Must be Linked to E7 DNA for Antitumor Effects. To assess whether the linkage of N-CRT to E7 is essential for the antitumor effect against E7-expressing tumors in vaccinated mice, we performed *in vivo* tumor protection experiments using TC-I tumor cells. Mice were vaccinated with mixtures of DNA constructs that encoded E7 DNA and separately, constructs encoding , N-CRT DNA, N-CRT/E7or N-CRT DNA. At the appropriate time, mice were challenged with E7-bearing TC-I tumor cells. All mice vaccinated with the N-CRT/E7 DNA vaccine remained tumor-free. In contrast, all mice vaccinated with the other DNA vaccine combinations (including a mixture of N-CRT DNA and E7 DNA) developed tumors within 2 weeks of challenge. This proves that linkage of N-CRT to E7 was required for the observed antitumor effects in vaccinated mice.

CD8+ T cells, but not CD4+ T cells or NK cells, are Important for the Antitumor effect Evoked by N-CRT/E7 DNA vaccine. *In vivo* antibody depletion experiments were conducted delete individual T cell subsets or NK cells. N-CRT/E7 DNA-vaccinated mice that had been depleted of CD8+ T cells, as well as all unvaccinated naive mice, grew tumors within 14 days. In contrast, all N-CRT/E7 DNA-vaccinated mice depleted of CD4+ T cells or of NK cells remained tumor free 56 days after challenge.

These results showed that suggest that in response to N-CRT/E7 DNA vaccine CD8+ T cell s, but not CD4+ T cells or NK cells, mediated the antitumor immunity.

Discussion

Thus, the present inventors demonstrated that linkage of DNA encoding N-CRT, P-CRT, or C-CRT to DNA encoding the HPV-16 E7 antigen can significantly enhance the potency of an E7-expressing DNA vaccine. Each domain of CRT linked with E7 DNA elicited strong E7-specific $CD8^+$ T cell immune responses, generated significant $CD8^+$ T cell-dependent protective effects against subcutaneous HPV-16 E7-expressing tumors, and could effectively treat lethal pulmonary tumor nodules. In comparison, vaccination with either N-CRT/E7 or CRT/E7 DNA significantly enhanced E7-specific antibody responses. Only DNA vaccines encoding the N domain inhibited angiogenesis leading to therapeutic independent of the immune response. Thus, a DNA vaccine encoding N-CRT (including full length CRT) linked to a tumor antigen represents a useful approach for treatment that combines immunotherapeutic and antiangiogenic approaches for increasing antitumor effects.

The mechanism for CRT (or CRT domain) immunological effects may include enhancing MHC class I processing of antigen by targeting linked antigen to the ER in transfected APCs such as DCs. Another possible mechanism for these effects is "cross-priming", whereby secretion of chimeric protein or lysis of cells expressing chimeric antigen releases the chimeric protein exogenously to be taken up and processed by other APCs via the MHC class I restricted pathways. CD91, an $\alpha 2$ macroglobulin receptor, serves as a receptor for heat shock proteins, including CRT, gp96, HSP70 and HSP90 and facilitate the cross-priming effects. There are still questions about which of the three CRT domains binds CD91. Both direct and cross-priming mechanisms may also explain the immunopotentiating effects or P-CRT/E7 DNA and C-CRT/E7 DNA.

Even though antibody-mediated responses are not considered to play an important role in controlling HPV-associated malignancies, antigen-specific Abs are significant in other tumor disease, such as the treatment of HER-2/neu antigen-bearing breast cancer cells. The chimeric N-CRT or CRT vaccine strategy described for E7 may be directly transferred to Her-2/neu epitopes to stimulate HER-2/neu-specific antibody responses that can arrest growth of cells expressing high levels of surface HER-2/neu (Harwerth, DVI *etal, Br J Cane 68:1* 140-5, 1993).

By inhibiting angiogenesis, including endothelial cell growth, N-CRT DNA, N-CRT/E7 DNA or N-CRT linked to any other antigen may be employed to reduce tumor neovascularization and inhibit tumor growth. N-CRT/E7 and CRT/E7 are two chimeric molecules that can control established tumors cells through E7-specific CD8+ T cell-mediated immune responses and inhibit the growth of tumor vasculature through antiangiogenesis. Effective antiangiogenic effects require repeated and high dose administration of DNA encoding CRT or N-CRT. A single DNA vaccination results in peak serum

CRT levels at 7 days post vaccination, which then taper to near-baseline levels within 14 days post vaccination (Xiao, F., *et al. Gene Ther 9:* 1207-13, 2002,). The level of serum CRT depends on the dose of CRT DNA given. Typical DNA vaccine doses (2μg) did not result in detectable serum CRT levels. Repeated, high-dose CRT DNA vaccination does lead to detectable levels of serum CRT and anti-angiogenic effects in vaccinated mice (Cheng *et al.,* 2001,m *supra).* Undesired side effects of angiogenesis inhibition include diminution of wound healing. However, this does not appear to occur at tumor-inhibiting doses (Lange-Asschenfeldt, B *et al, J Invest Dermatol* 777:1036-41, 2001). Furthermore, additional experiments by the present inventors showed that vaccination with the CRT/E7 DNA vaccine did not inhibit wound healing or cause any pathologic changes in the major organs of mice.

All references cited above are all incorporated by reference herein, in their entirety, whether specifically incorporated or not. All publications, patents, patent applications, GenBank sequences and ATCC deposits, cited herein are hereby expressly incorporated by reference for all purposes.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

CLAIMS

1. A nucleic acid molecule that is an expression vector expressable in a eukaryotic cell, and encodes a chimeric or fusion polypeptide, which molecule comprises:

(a) a first nucleic acid sequence encoding a first polypeptide which is calreticulin (CRT) or a biologically active homologue, domain or fragment thereof,

which homologue, domain or fragment (i) forms complexes with peptides *in vitro;* (ii) when expressed in a cell, participates in folding and assembly of nascent glycoproteins, (iii) when expressed in a cell, associates with peptides transported into the endoplasmic reticulum by transporters that are associated with antigen processing, and/or (iv) inhibits angiogenesis;

(b) a second nucleic acid sequence that is linked in frame to said first nucleic acid sequence and that encodes an antigenic polypeptide or peptide; and

(c) operably linked thereto, a promoter active in said eukaryotic cell and, optionally, one or more regulatory elements that enhance expression of said nucleic acid in said cell.

2. The nucleic acid molecule of claim 1, wherein the antigenic peptide comprises an epitope that binds to a MHC class I protein.

3. The nucleic acid molecule of claim 1, wherein the first polypeptide is encoded by SEQ ID NO: 10 or a fragment thereof that encodes a biologically active domain or fragment of said polypeptide.

4. The nucleic acid molecule of claim 3, wherein the first nucleic acid sequence encodes one or more CRT fragments or domain selected from the group consisting of (a) N-CRT, (b) P-CRT, (c) S-CRT and (d) a biologically active variant of (a), (b) or (c) but does not encode full length CRT.

5. The nucleic acid molecule of claim 4, wherein the first nucleic acid sequence encodes N-CRT (SEQ ID NO: 14), P-CRT (SEQ ID NO: 15), S-CRT (SEQ ID NO: 16) or a homologue of N-CRT, P-CRT or S-CRT.

6. The nucleic acid molecule of claim 4, wherein the first nucleic acid sequence encodes N-CRT (SEQ ID NO: 14).

7. The nucleic acid molecule of claim 4, wherein the first nucleic acid sequence encodes any two or more of N-CRT (SEQ ID NO: 14), P-CRT (SEQ ED NO: 15), C-CRT (SEQ ID NO:16)or any combination thereof.

8. The nucleic acid molecule of claim 1 wherein the antigen is one which is present on, or cross-reactive with an epitope of, a pathogenic organism, cell, or virus.

9. The nucleic acid molecule of claim 8, wherein the virus is a human papilloma virus.

10. The nucleic acid molecule of claim 9, wherein the antigen is an E7 or E6 polypeptide of HPV-16 having the sequence SEQ ID NO:3 or SEQ ID NO:6, respectively, an -in-frame linked combination of E6 and E7, an antigenic fragment of E6 or E7, or non-oncogenic mutant or variant of E6 or E7; or an in-frame linked combination of a non-oncogenic mutant or variant of E6 and of E7.

11. The nucleic acid molecule of claim 10, wherein the HPV-16 E7 polypeptide is a non-oncogenic mutant or variant of said E7 polypeptide.

12. The nucleic acid molecule of claim 9 wherein the sequence of the non-oncogenic mutant or variant E7 polypeptide differs from SEQ ID NO:3 by one or more of the following substitutions:

(a) Cys at position 24 to GIy or Ala;

(b) GIu at position 26 to GIy or Ala; and

(c) Cys at position 9 1 to GIy or Ala.

13. The nucleic acid molecule of claim of claim 12 wherein the sequence of the non-oncogenic mutant or variant E7 polypeptide is sequence SEQ ID NO:4.

14. The nucleic acid molecule of claim 10, wherein the antigen is the E6 polypeptide having the sequence SEQ ID NO: 6 or an antigenic fragment thereof.

15. The nucleic acid molecule of claim 11, wherein the HPV-16 E6 polypeptide is a non-oncogenic mutant or variant of said E6 polypeptide.

16. The nucleic acid molecule of claim 15 wherein the sequence of the non oncogenic mutant or variant E6 polypeptide differs from SEQ ID NO:6 by one or more of the following substitutions:

(a) Cys at position 63 to GIy or Ala;

(b) Cys at position 106 to GIy or Ala; and

(c) He at position 128 to Thr.

17. The non oncogenic mutant E6 polypeptide of claim 15 having the sequence SEQ ID NO:7.

18. The nucleic acid molecule of claim 10 wherein the antigen is a linked, in-frame combination of E7 and E6 polypeptide, an antigenic fragment thereof, or a non-oncogenic mutant or variant of E7 and E6.

19. The nucleic acid molecule of any of claims 1-18 that is part of a plasmid.

20. The nucleic acid molecule of claim 19 wherein said plasmid is pNGV4a.

21. The nucleic acid molecule of claim 1 that is characterized as pNGVL4a/CRT/E7(detox), and has the sequence SEQ ID NO:20.

22. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) a composition comprising the nucleic acid molecule of any of claims 1-18.

23. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) the nucleic acid molecule of claim 19.

24. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) the nucleic acid molecule of claim 20.

25. A pharmaceutical composition capable of inducing or enhancing an antigen-specific immune response, comprising:

(a) pharmaceutically and immunologically acceptable excipient in combination with;

(b) the nucleic acid molecule of claim 21.

26. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 22, thereby inducing or enhancing said response.

27. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 23, thereby inducing or enhancing said response.

28. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 24, thereby inducing or enhancing said response.

29. A method of inducing or enhancing an antigen specific immune response in a subject comprising administering to the subject an effective amount of the pharmaceutical composition of claim 25, thereby inducing or enhancing said response.

30. The method of claim 26, wherein the response is mediated at least in part by CDS^+ cytotoxic T lymphocytes (CTL).

31. The method of claim 29, wherein the response is mediated at least in part by $CD8^+$ cytotoxic T lymphocytes (CTL).

32. The method of claim 26 wherein said subject is a human.

33. The method of claim 27 wherein said subject is a human.

34. The method of claim 28 wherein said subject is a human.

35. The method of claim 29 wherein said subject is a human.

36. The method of claim 26 wherein said administering is by a intramuscular injection by gene gun administration or by needle-free jet injection.

37. The method of claim 27 wherein said administering is by a intramuscular injection, by gene gun administration or by needle-free jet injection.

38. The method of claim 28 wherein said administering is by a intramuscular injection, by gene gun administration or by needle-free jet injection.

39. The method of claim 29 wherein said administering is by a intramuscular injection, by gene gun administration or by needle-free jet injection.

39. The method of claim 29 wherein said administering is by a intramuscular injection, by gene gun administration or by needle-free jet injection.

40. The method of claim 39 wherein said administering is by a intramuscular injection.

41. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 22, wherein said second nucleic acid sequence encodes one or more epitopes of E7 or E6, respectively, thereby inhibiting said growth or preventing said re-growth.

42. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 23, wherein said second nucleic acid sequence encodes one or more epitopes of E7 or E6, respectively, thereby inhibiting said growth or preventing said re-growth.

43. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 24, wherein said second nucleic acid sequence encodes one or more epitopes of E7 or E6, respectively, thereby inhibiting said growth or preventing said re-growth.

44. A method of inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject, comprising administering to said subject an effective amount of a pharmaceutical composition of claim 25, wherein said second nucleic acid sequence encodes one or more epitopes of E7 or E6, respectively, thereby inhibiting said growth or preventing said re-growth.

45. Use of a nucleic acid molecule according to any one of claims 1 to 18, for the manufacture of a medicament for inducing or enhancing an antigen specific immune response in a subject.

46. Use of a nucleic acid molecule according to claim 19, for the manufacture of a medicament for inducing or enhancing an antigen specific immune response in a subject.

47. Use of a nucleic acid molecule according to any one of claims 21, for the manufacture of a medicament for inducing or enhancing an antigen specific immune response in a subject.

48. Use of a nucleic acid molecule according to any one of claims 1 to 18, for the manufacture of a medicament for inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject.

49. Use of a nucleic acid molecule according to claim 19, for the manufacture of a medicament for inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject.

50. Use of a nucleic acid molecule according to any one of claims 21, for the manufacture of a medicament for inhibiting growth or preventing re-growth of a tumor expressing HPV E7 or E6 protein in a subject.

51. Use according to any one of claims 45-50 wherein the subject is a human.

52. Use according to any one of claims 45-50 wherein the medicament is administered to the subject by intramuscular injection, by gene gun administration or by needle-free jet injection.

53. Use according to claim 51 wherein the medicament is administered to the subject by intramuscular injection.

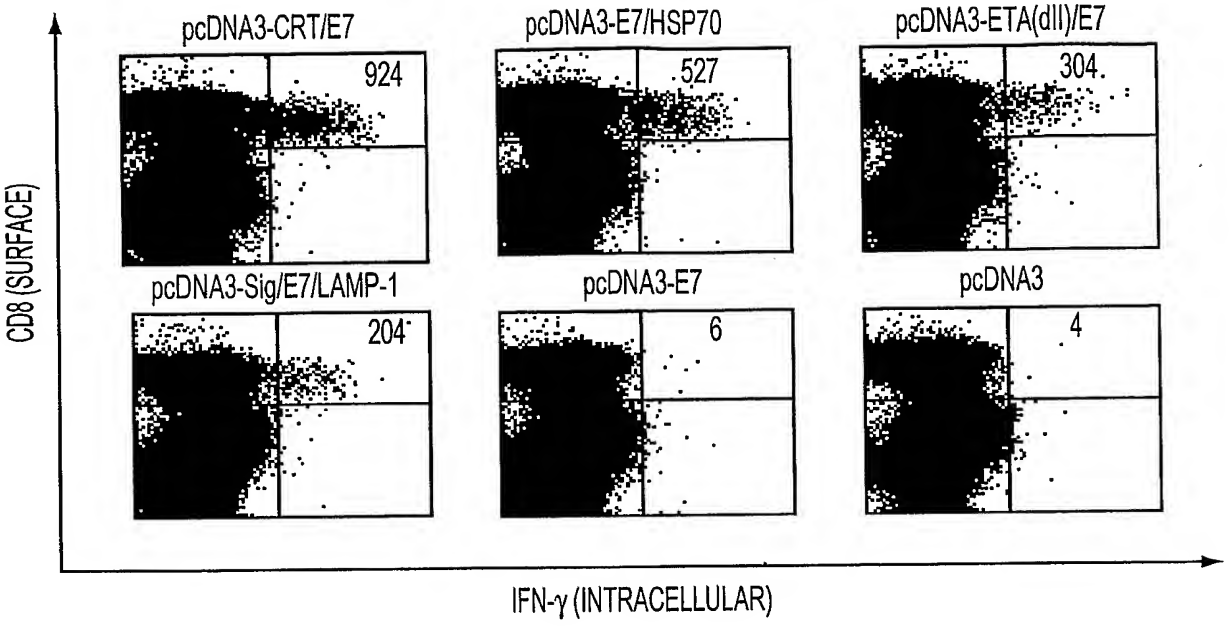
pcDNA3-CRT/E7
924
pcDNA3-E7/HSP70
527
pcDNA3-ETA(dII)/E7
304
pcDNA3-Sig/E7/LAMP-1
204
pcDNA3-E7
6
pcDNA3
4
CD8 (SURFACE)
IFN-γ (INTRACELLULAR)

FIG. 1

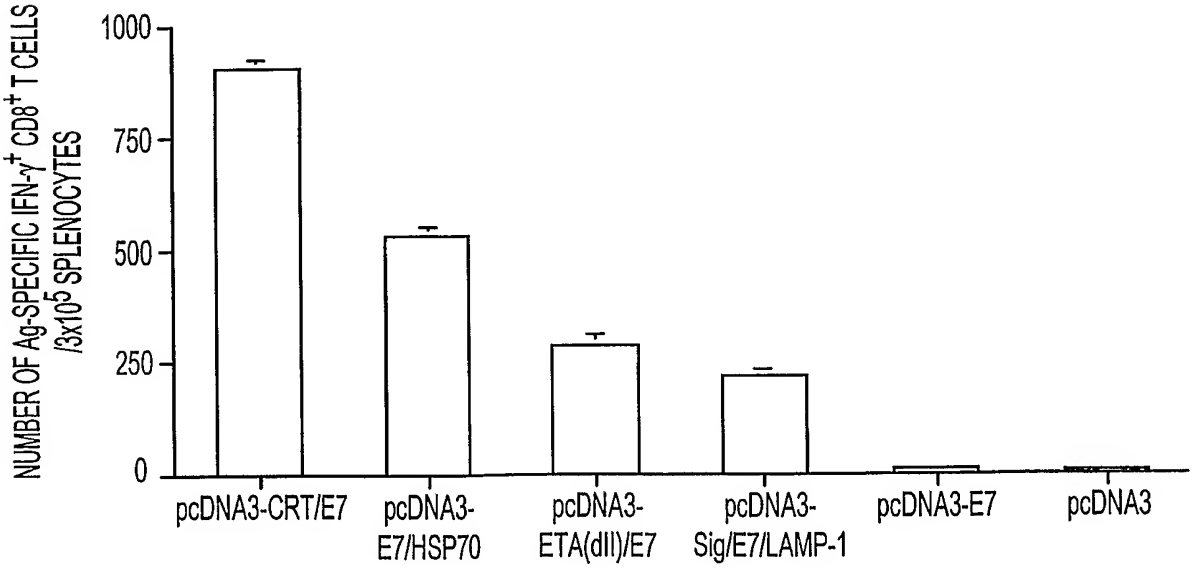
NUMBER OF Ag-SPECIFIC IFN-γ+ CD8+ T CELLS /3x10^5 SPLENOCYTES
1000
750
500
250
0
pcDNA3-CRT/E7
pcDNA3-E7/HSP70
pcDNA3-ETA(dII)/E7
pcDNA3-Sig/E7/LAMP-1
pcDNA3-E7
pcDNA3

FIG. 2

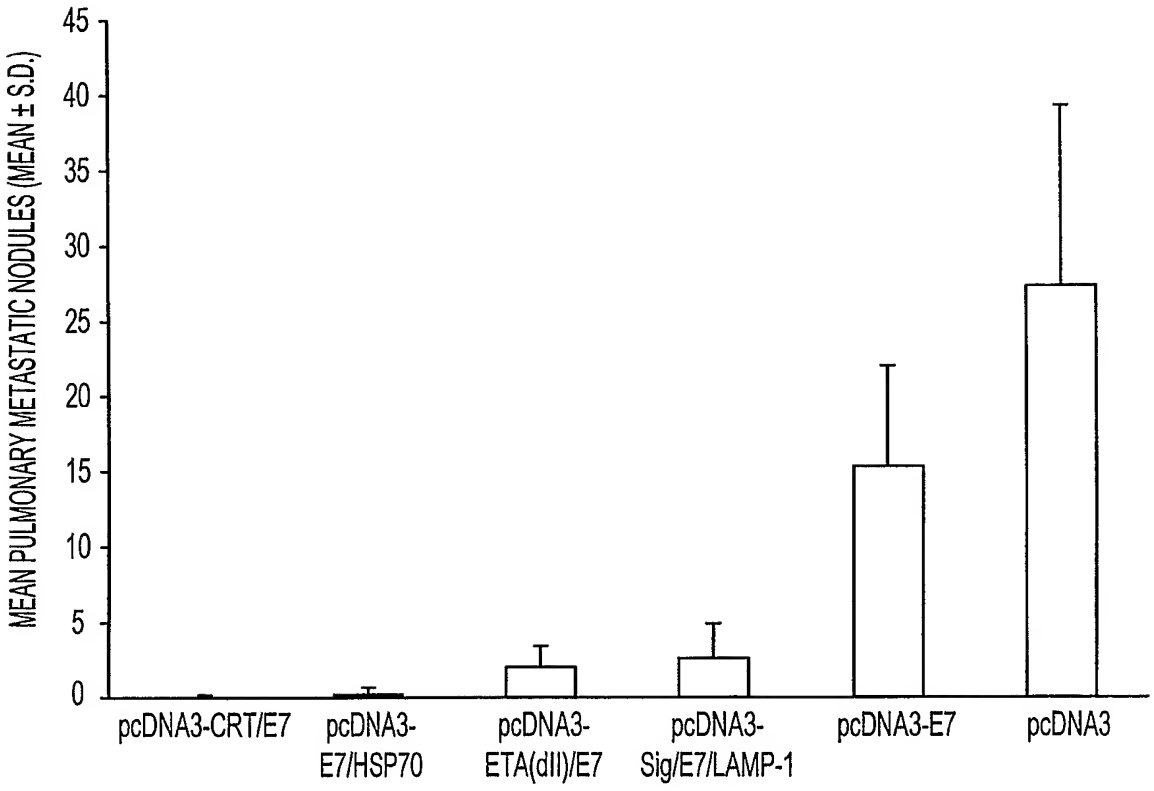
MEAN PULMONARY METASTATIC NODULES (MEAN ± S.D.)
45
40
35
30
25
20
15
10
5
0
pcDNA3-CRT/E7
pcDNA3-E7/HSP70
pcDNA3-ETA(dII)/E7
pcDNA3-Sig/E7/LAMP-1
pcDNA3-E7
pcDNA3

FIG. 3

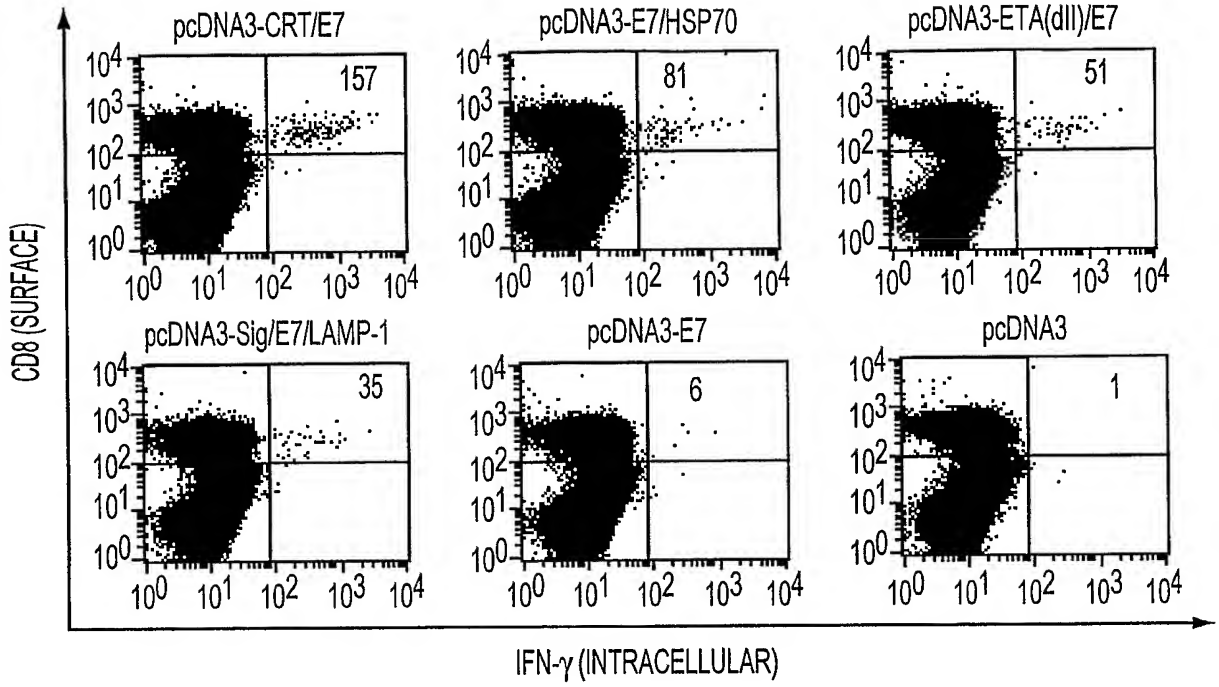
pcDNA3-CRT/E7
157
pcDNA3-E7/HSP70
81
pcDNA3-ETA(dII)/E7
51
pcDNA3-Sig/E7/LAMP-1
35
pcDNA3-E7
6
pcDNA3
1
CD8 (SURFACE)
IFN-γ (INTRACELLULAR)

FIG. 4

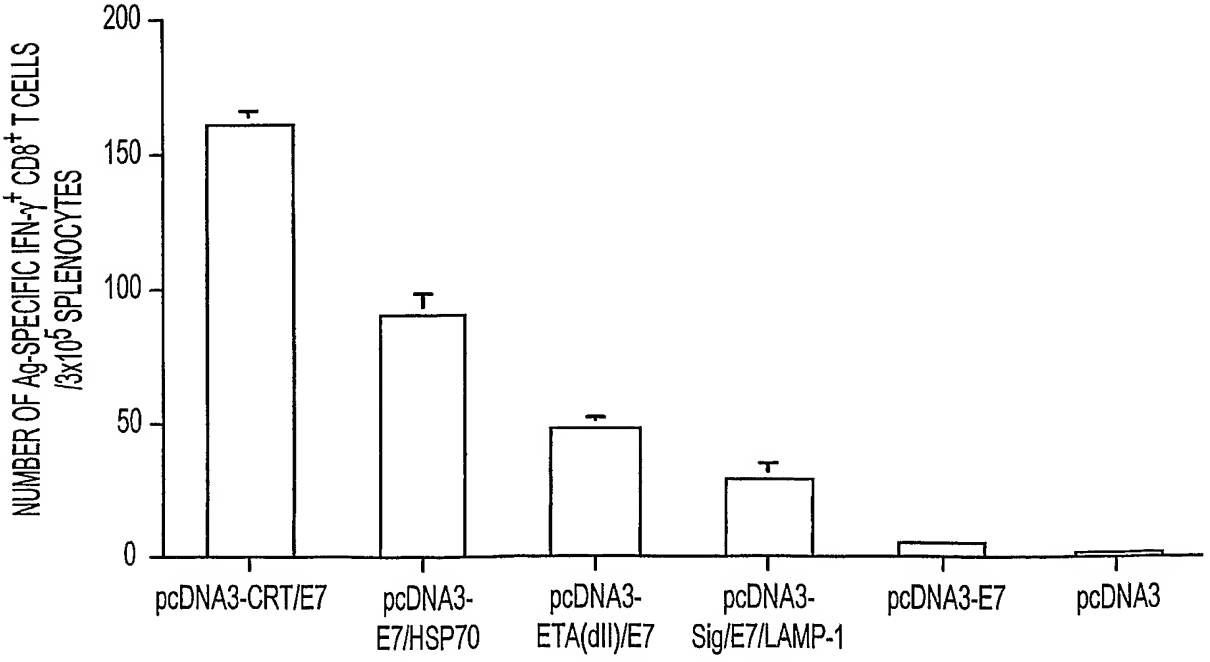
NUMBER OF Ag-SPECIFIC IFN-γ+ CD8+ T CELLS /3x10^5 SPLENOCYTES
200
150
100
50
0
pcDNA3-CRT/E7
pcDNA3-E7/HSP70
pcDNA3-ETA(dII)/E7
pcDNA3-Sig/E7/LAMP-1
pcDNA3-E7
pcDNA3

FIG. 5

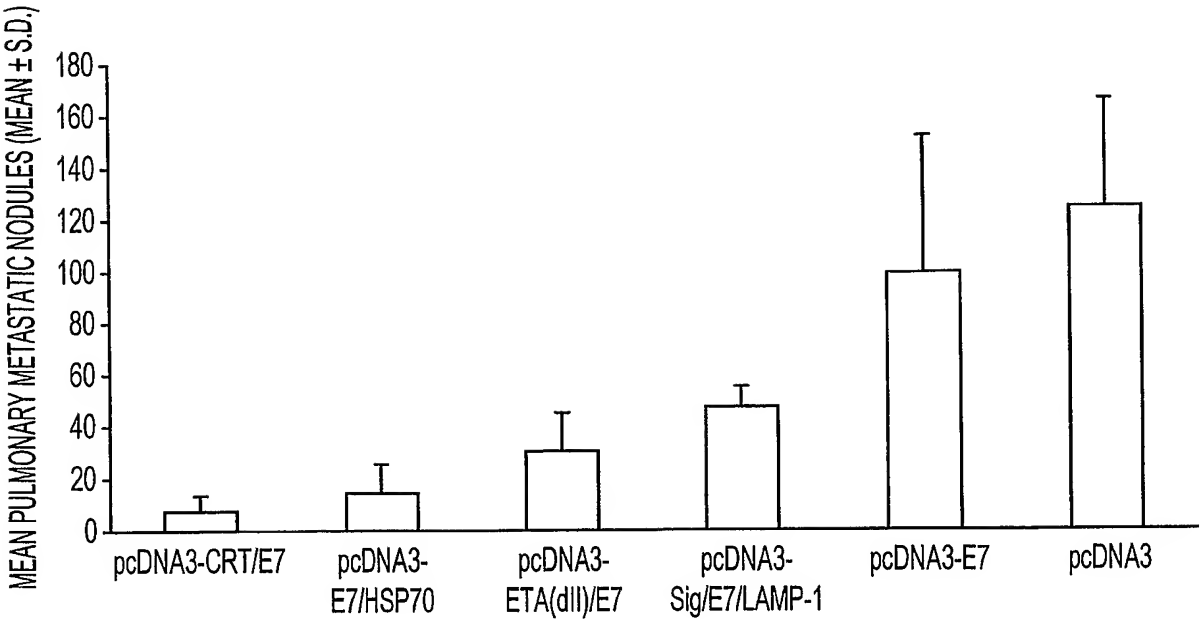
MEAN PULMONARY METASTATIC NODULES (MEAN ± S.D.)
180
160
140
120
100
80
60
40
20
0
pcDNA3-CRT/E7
pcDNA3-E7/HSP70
pcDNA3-ETA(dII)/E7
pcDNA3-Sig/E7/LAMP-1
pcDNA3-E7
pcDNA3

FIG. 6

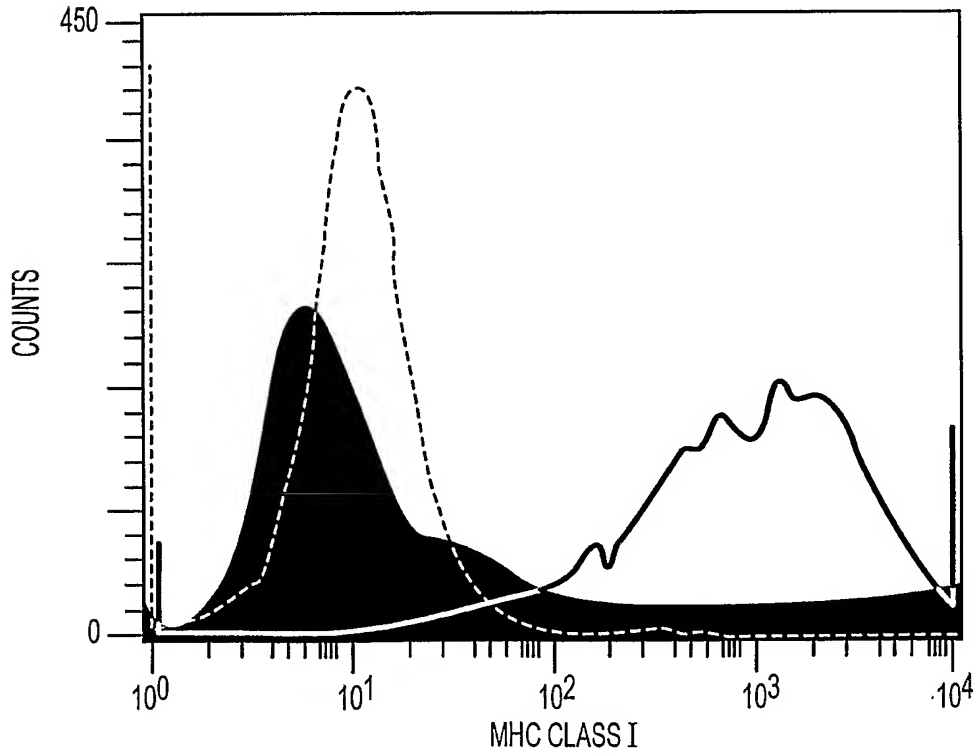
450
COUNTS
0
10^0
10^1
10^2
10^3
10^4
MHC CLASS I

FIG. 7

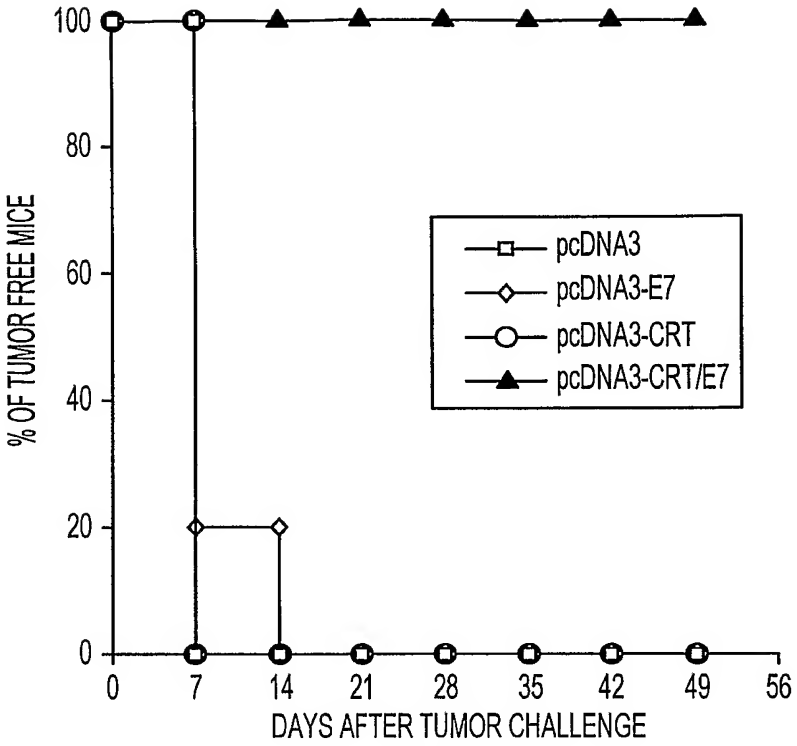
100
80
60
40
20
0
% OF TUMOR FREE MICE
0
7
14
21
28
35
42
49
56
DAYS AFTER TUMOR CHALLENGE
pcDNA3
pcDNA3-E7
pcDNA3-CRT
pcDNA3-CRT/E7

FIG. 8

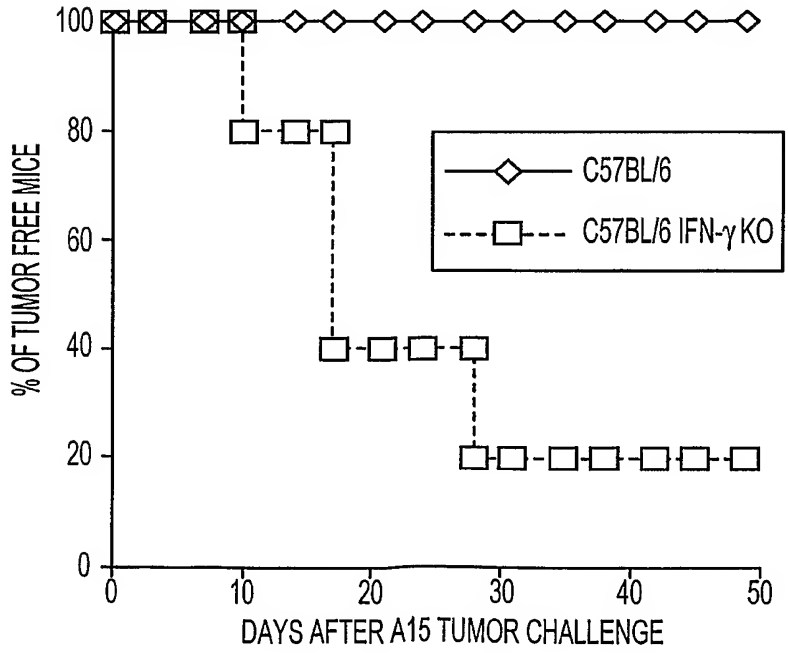
100
80
60
40
20
0
% OF TUMOR FREE MICE
0
10
20
30
40
50
DAYS AFTER A15 TUMOR CHALLENGE
C57BL/6
C57BL/6 IFN-γ KO

FIG. 9

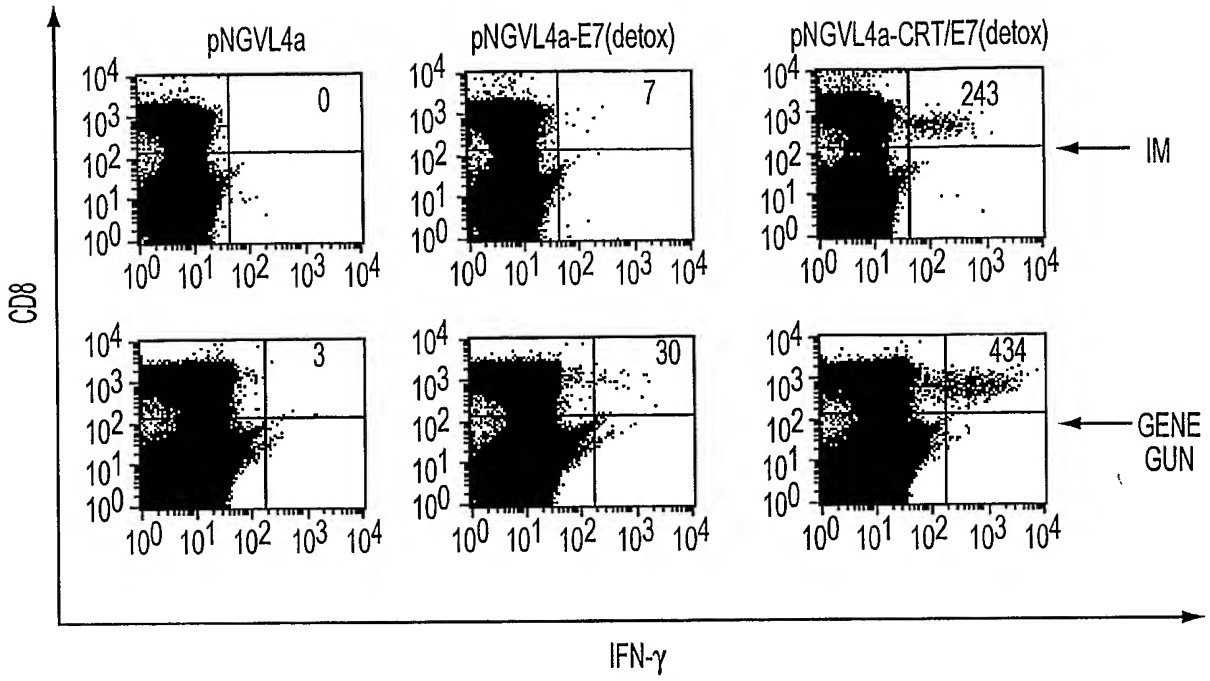
pNGVL4a
pNGVL4a-E7(detox)
pNGVL4a-CRT/E7(detox)
0
7
243
3
30
434
IM
GENE GUN
CD8
IFN-γ

FIG. 10

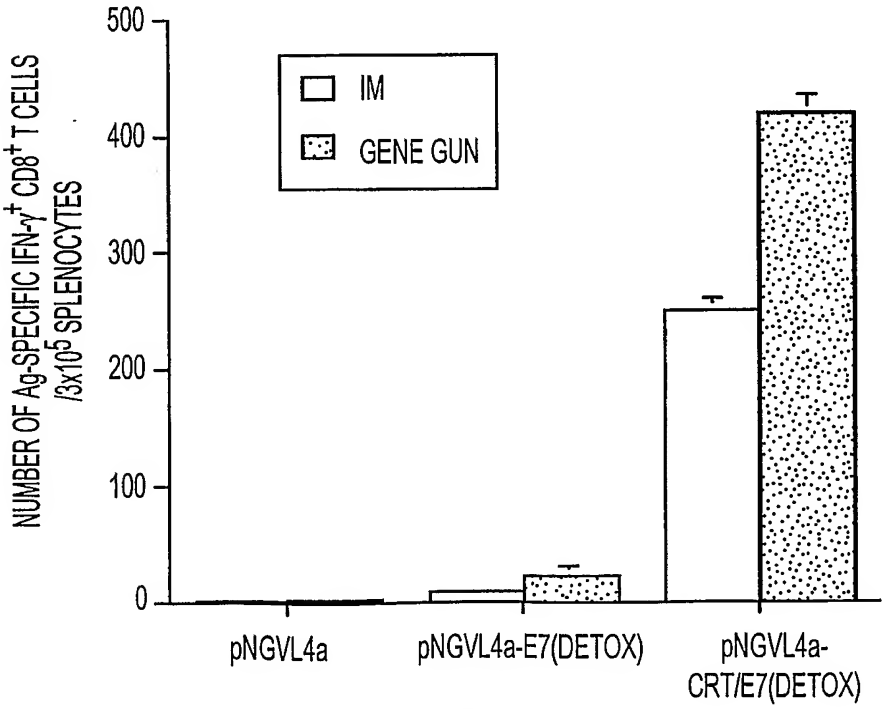
IM
GENE GUN
NUMBER OF Ag-SPECIFIC IFN-γ+ CD8+ T CELLS /3x10^5 SPLENOCYTES
500
400
300
200
100
0
pNGVL4a
pNGVL4a-E7(DETOX)
pNGVL4a-CRT/E7(DETOX)

FIG. 11

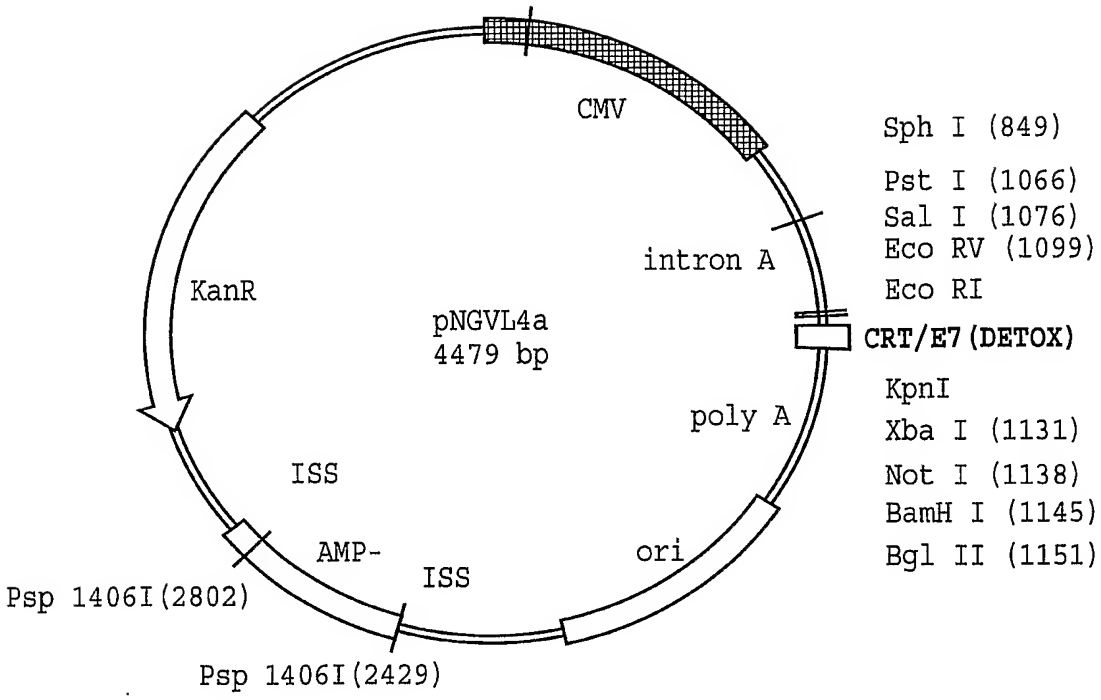
CMV
Sph I (849)
Pst I (1066)
Sal I (1076)
Eco RV (1099)
intron A
Eco RI
KanR
pNGVL4a
4479 bp
CRT/E7(DETOX)
KpnI
poly A
Xba I (1131)
Not I (1138)
ISS
BamH I (1145)
Bgl II (1151)
AMP-
ori
ISS
Psp 1406I(2802)
Psp 1406I(2429)

FIG. 12